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NEW CHEMICAL MODELS FOR CARBONIC ANHYDRASE

by

© JOSÉ LUIS COCHO

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled NEW CHEMICAL MODELS FOR CARBONIC ANHYDRASE
submitted by JOSÉ LUIS COCHO in partial fulfilment of the requirements for the degree of Master of Science.

"Though this be madness,
yet there is method in't"

Hamlet [II(2)]

DEDICATION

A mis padres.

ABSTRACT

One of the most successful models mimicking the physical properties and catalytic activity of carbonic anhydrase is tris-(4,5-di-iso-propyl-2-imidazolyl) phosphine:Zn^{2+}. The present work deals with the synthesis and physical studies of three analogous compounds:
1) bis-(4,5-di-iso-propyl-2-imidazolyl)-2-imidazolyl-phosphine:Zn^{2+} (5:Zn^{2+}) 2) bis-(4,5-di-iso-propyl-2-imidazolyl)-4(5)-hydroxyethyl-2-imidazolyl phosphine:Zn^{2+} (6:Zn^{2+}) and 3) tris-(4,5-di-n-propyl-2-imidazolyl) phosphine:Zn^{2+} (7:Zn^{2+}).

The aforementioned compounds were prepared by means of a nucleophilic displacement of the corresponding 2-imidazolyl anions on phosphorous trichloride and their Zn^{2+} and Co^{2+} complexes were studied as active site models for carbonic anhydrase. In order to be considered good models for the active site of carbonic anhydrase the ligands must be shown to bind Zn^{2+} or Co^{2+} strongly in a tridentate fashion,therefore, the metal binding ability of the compounds was assayed using a potentiometric titration. All of them proved to bind Zn^{2+} better than Co^{2+}, a property that is shared with the native enzyme.

NMR studies of the ligands as a function of [Zn^{2+}] were performed. From these it was found that the three

compounds exist as 1:1 complexes and that two of them (5:Zn^{2+} and 6:Zn^{2+}) undergo a dynamic exchange on the NMR timescale indicative of an imidazole debinding, tautomerization and rebinding through the opposite nitrogen.

The coordination number around the metal was indicated via the UV-visible spectra of the Co^{2+} complexes. While some 4 or 5 coordination was observed for the Co(II) complexes of 5 and 6 only 7 showed a predominant amount of this. The effect of different anions on the coordination number was assayed by studying the UV-visible spectra of the Co^{2+} complexes in the presence of added anions. A strong dependence of the coordination numbers with the different anions was found (Cl^- affording the largest amounts of 4 or 5 coordination).

Finally, the catalytic activity was assayed from the direction of bicarbonate dehydration. The three ligands proved to be active but this activity is small when compared to the enzyme. The compound that afforded maximum activity was 7:Zn^{2+} followed by 5:Zn^{2+} and then 6:Zn^{2+}. The effect of inhibitory anions was studied by adding these to the solution containing the compound under study. Cl^- proved to be the best inhibitor, however, compounds 5:Zn^{2+} and 7:Zn^{2+} still showed activity even in the presence of large amounts of added anion.

All of the previous experiments afforded values for k_{obs} which is a summation of k_f and k_r for the reaction under study ($HCO_3^- \underset{k_r}{\overset{k_f}{\rightleftharpoons}} CO_2$). A saturation phenomenon in the equilibration rate was observed as a function of increasing [$NaHCO_3$], but the system proved too complicated to derive a complete rate expression. Thus, an attempt was made to perform initial rate experiments. These were undertaken from both the direction of bicarbonate dehydration and carbon dioxide hydration, but problems arose due to an unexpected interaction between indicator and bicarbonate.

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CHAPTER I

INTRODUCTION

I - A CARBONIC ANHYDRASE

Carbonic anhydrase[1] is a widely distributed enzyme that catalyses the interconversion of carbon dioxide and bicarbonate at a rate which is one of the fastest known in enzyme catalysis, the maximum turnover number being about 10^6 s^{-1} at 25°C. The enzyme has also been found to catalyze the hydrolysis of certain esters and the hydration of aldehydes, although this is not presently believed to be its physiological role.

The enzyme was first reported by Meldrum and Roughton in 1932[2]; a year later their finding was confirmed by Stadie and O'Brien[3]. The properties of the enzyme were studied as early as 1933[4] and by 1939 it was shown that it was a zinc-containing enzyme[5] thus demonstrating the first physiological function for this metal ion.

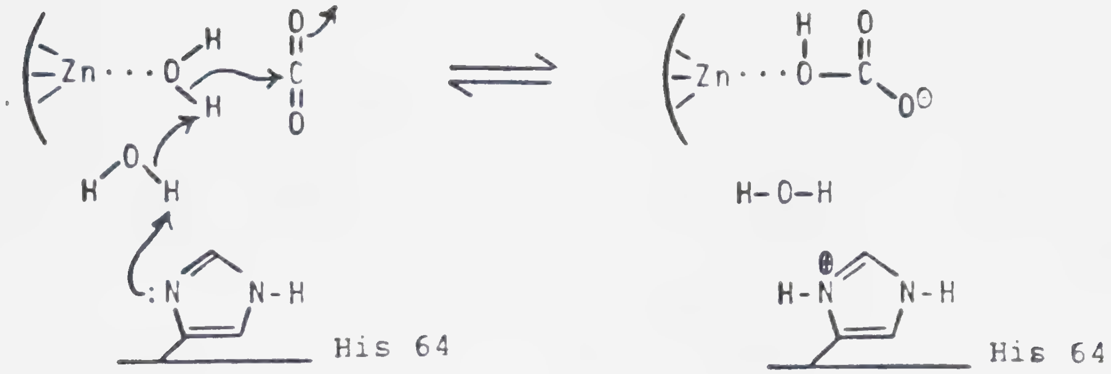
I - B STRUCTURE

Purification of the human erythrocyte enzyme has shown that human red cells contain two forms of carbonic anhydrase designated as B and C and differing in catalytic properties[6]. Most studies of carbonic anhydrase have been made on enzymes obtained from human and bovine erythrocytes. The mammalian enzymes are single chain proteins with a molecular weight of around 30,000,[1]

and are elipsoidal in shape, about 41x42x55 Å in dimension with a number of cavities. The essential zinc ion is located in a 12 Å deep conical cavity with an opening about 10 Å in diameter[7]. This active site cavity is identified by the presence of the zinc ion and by the fact that inhibitors bind there[8].

As with other enzymes such as liver alcohol dehydrogenase (LADH) and lactate dehydrogenase, the active site in carbonic anhydrase is arranged in a beta or pleated sheet structure and not in an alfa helix configuration[9]. The packing of the side chains of the carbonic anhydrase follows the general rule of "hydrophobic in and hydrophilic out" as in all other globular proteins observed so far[10].

Human carbonic anhydrase C has five histidine residues within 8 Å of the active site zinc atom at positions 64,94,96,107 and 119, with those at positions 94,96 and 119 being ligands to the active site zinc atom[11]. The catalytic significance of histidines 64 and 107 is not known, however, modifying His-64 with bromopyruvic acid yields an enzyme with about 30% retained activity which has led to the suggestion that His-64 could play an important role as a proton transfer agent[12]. (Eq. 1)



(1)

The zinc ion has a fourth ligand that has been identified as a solvent molecule resulting in a distorted tetrahedral geometry[10] about the metal. Based on the circular dichroism spectra of the Co(II) derivative of the enzyme, some groups have suggested that a five coordinate geometry around the high spin Co(II) ion in the alkaline form of the enzyme cannot be ruled out[13]. The possibility exists that the ionization at the active site is associated with a shift from 4 to 5 coordination, the latter being the active species for CO_2 hydration [14,15].

The water molecule directly ligated to the metal ion is said to be hydrogen bonded to threonine 199 and the fact that this amino acid residue forms hydrogen bonds with most inhibitors suggests that it plays an active role in catalysis[16].

Since the zinc ligands are neutral and bind to the metal through coordinate covalent bonds, the charge of the zinc ion has been suggested to play a role in the high catalytic efficiency of carbonic anhydrase[17]. Zn^{2+}

can be removed by dialysis of the enzyme at low pH with 1,10-phenanthroline or at high pH with 2,3-dimercaptopropanol[10]. The apoenzyme thus obtained is devoid of any catalytic power. The activity is wholly restored by addition of Zn^{2+} and partly restored when Co^{2+},Mn^{2+} or Cd^{2+} are bound to the apoenzyme. Other metals have been substituted in carbonic anhydrase but no activity has been observed[18]. Each of these metals binds at the same position as Zn^{2+} [10]. It has been suggested that the only properties common to the metals that render an active enzyme are the flexibility of their coordination[19] and the charge in the ion[17].

I - C INHIBITION

The carbonic anhydrases are inhibited by monovalent anions, the strength of inhibition following the Hofmeister series which corresponds to the order of increasing negative hydration energies[20]. Anions can be listed according to their inhibitory power as follows:

$SH^- > CN^- > NCO^- > SCN^- > N_3^- > I^- > HCO_3^- > B_r^- > CH_3CO_2^- > Cl$

Recent work by Y. Pocker indicates that at pH's higher than 7, the mode of anion inhibition changes sharply from competitive to uncompetitive for anions that have no labile hydrogen[21]. Therefore the bicarbonate ion behaves differently following a "linear uncompetitive"

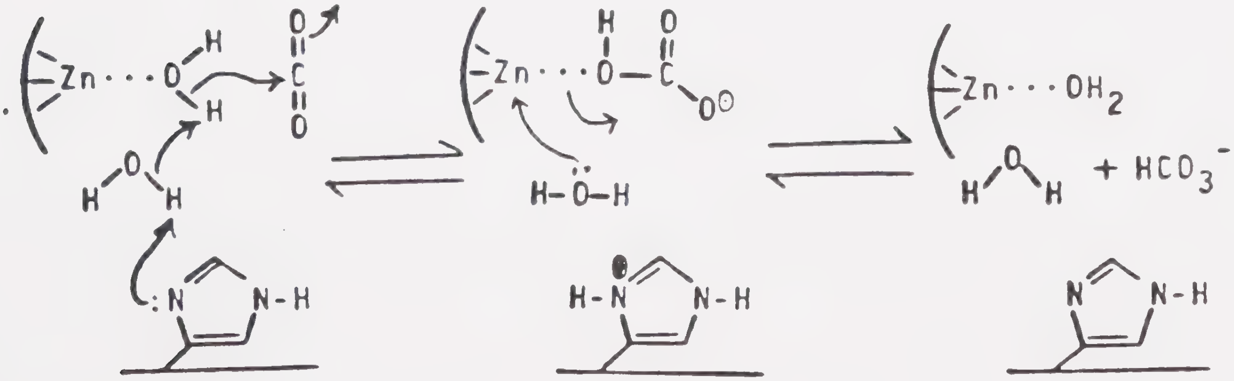
mechanism in which the inhibitory anion could bind to a fifth coordination site on the Zn^{2+} ion.

I - D MECHANISM OF ACTION

Although carbonic anhydrase was initially purified more than twenty years ago and the reaction it catalyses is the formally simple hydration of carbon dioxide, work by many groups has failed to produce a generally accepted mechanism for its action.

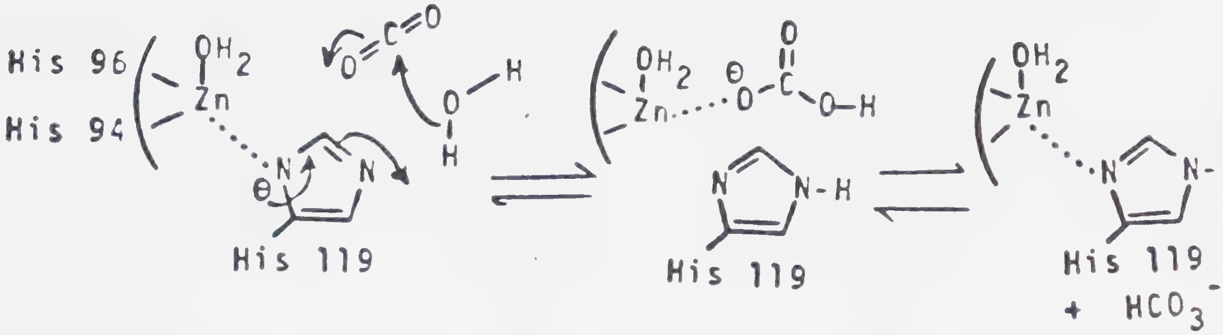
The activity of carbonic anhydrase around neutral pH is governed by the ionization of a group with a pKa near 7. Two major proposals concerning the identity of this group have been: 1) that its basic form represents either the imidazole moiety of a histidine residue, indirectly or directly linked to the essential metal ion[19], or 2) a zinc coordinated hydroxide ion[22]. With this in mind several mechanisms of action have been proposed. Among them are the following: (metal ion charges omitted for simplicity).

1) General base assisted attack of $Zn\text{-}OH_2$[23]. (Eq. 2)



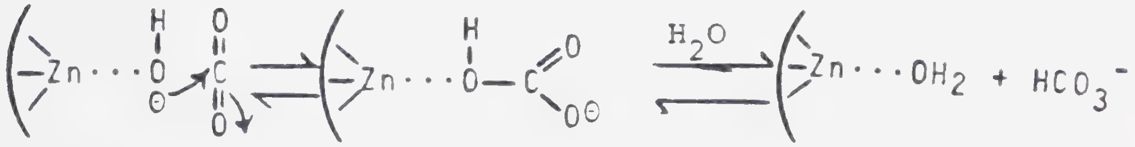
(2)

2) General base attack by H_2O assisted by His-119[24]. (Eq. 3)


His 96
His 94
His 119
His 119
His 119
+ HCO_3^-

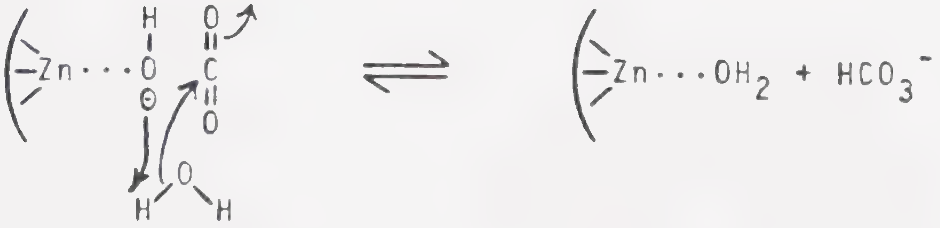
(3)

3) Nucleophilic attack by ZnOH[25]. (Eq. 4)



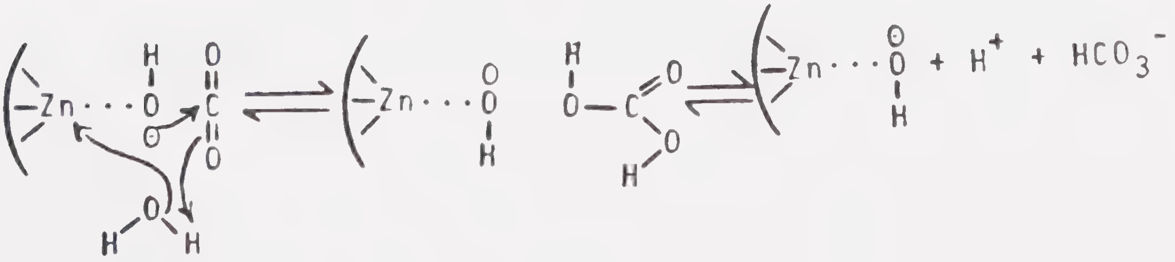
(4)

4) General base attack by H_2O assisted by ZnOH[26]. (Eq. 5)



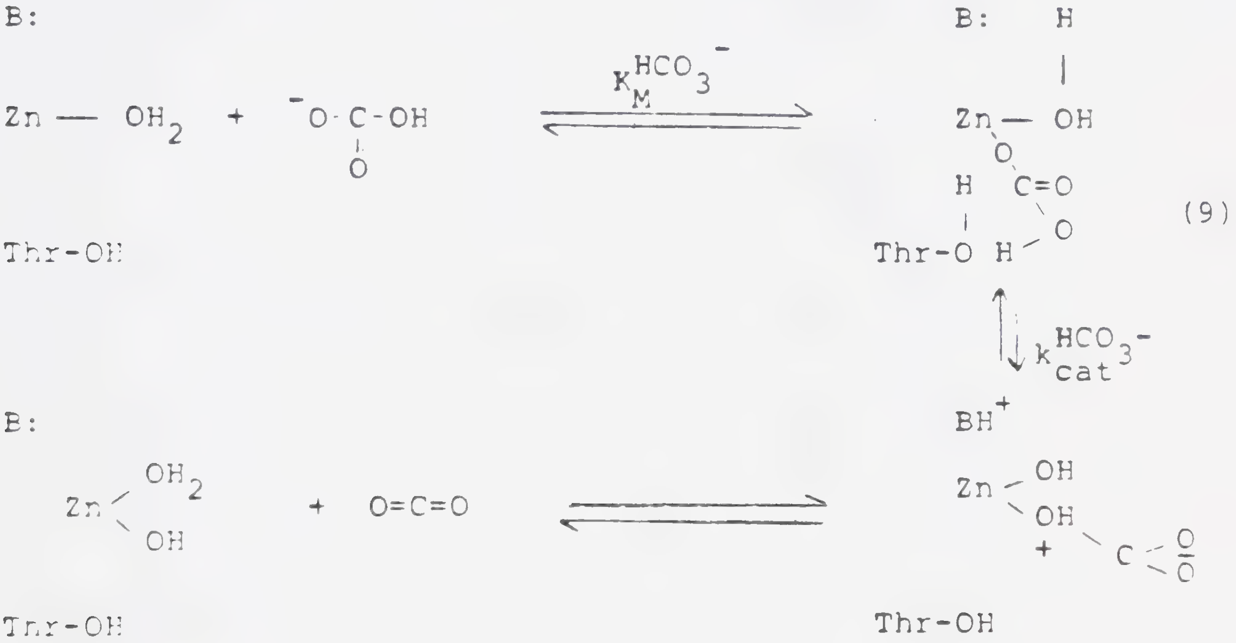
(5)

5) Nucleophilic attack by ZnOH with concomitant proton transfer[27]. (Eq. 6)



(6)

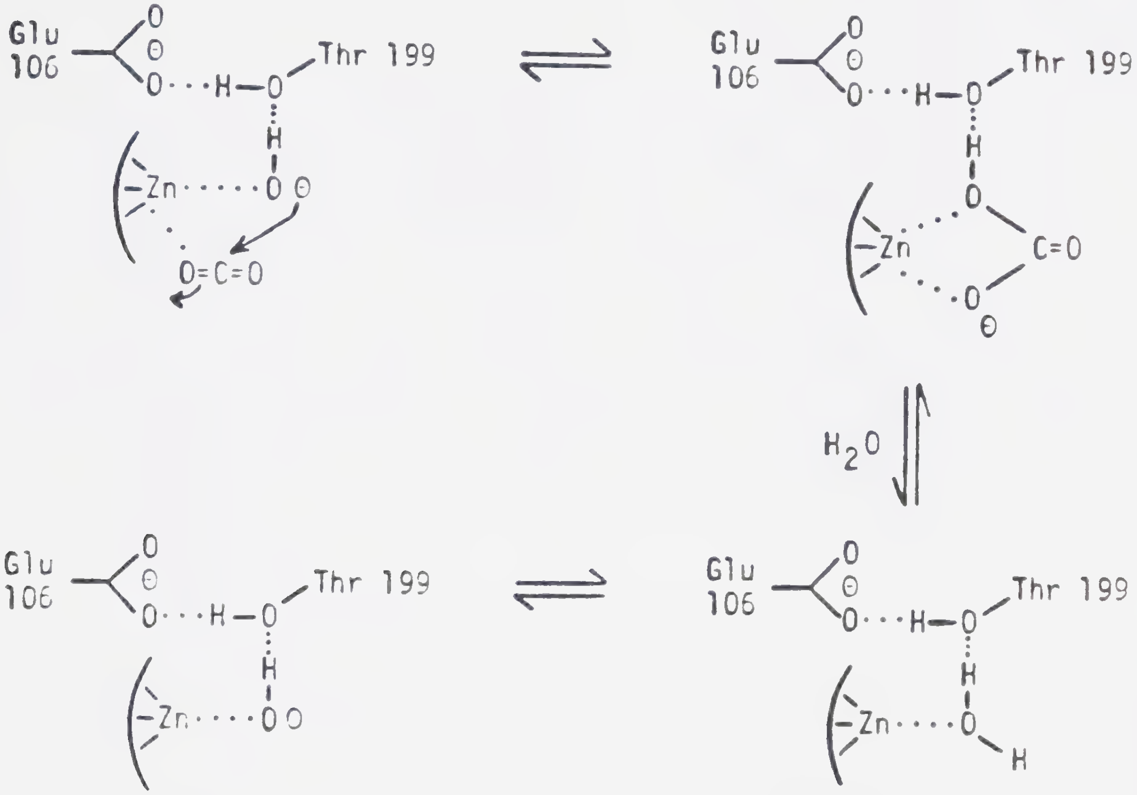
Pocker and Deits[28] have recently put forth a mechanism that involves a non-ligand histidine near the Zn^{2+} ion, a threonine and the bicarbonate proton as catalyst. Eq. 9 illustrates the mechanism (B represents the active site histidine as well as any additional intervening H_2O molecules that may participate.)


B:
Zn — OH_2
+
^-O-C-OH
O
Thr-OH
$K_M^{HCO_3^-}$
B: H
Zn — OH
O
H C=O
O
Thr-O H
(9)
$k_{cat}^{HCO_3^-}$
B:
Zn
OH_2
OH
Thr-OH
+
O=C=O
BH^+
Zn
OH
OH
+
C
O
O
Thr-OH

The enzyme ends up in its basic form with a proton transfer step from surrounding solvent or buffer to reprotonate the enzyme and loss of a water from the Zn^{2+} completing the HCO_3^- catalytic cycle.

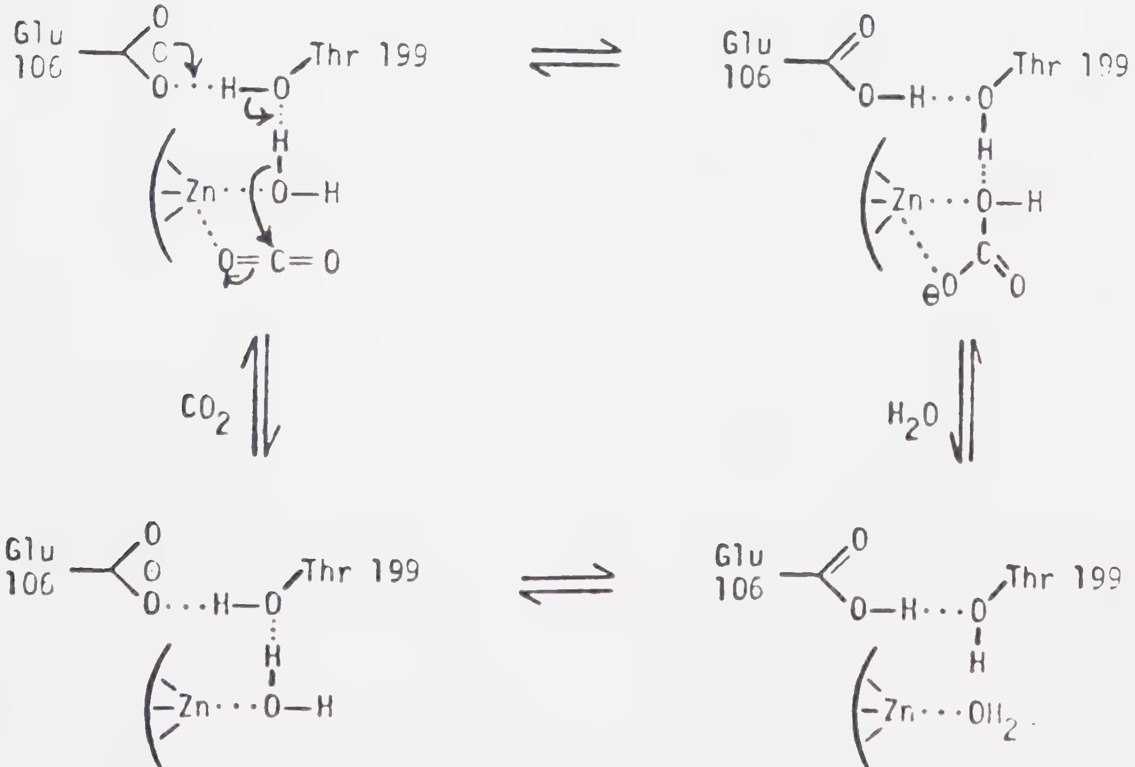
Pesando and Gupta[29] have disagreed with mechanisms that involve a Zn-bound water molecule at the active site as the ionizing group at pH 7 and identified the group controlling catalytic activity as an active site histidine.

6) Nucleophilic attack by ZnOH assisted by hydrogen bonding with Thr 199 and Glu 106[16]. (Eq. 7)


Glu 106
Thr 199
Zn
O=C=O
C=O
H2O

(7)

7) General base attack by $ZnOH_2$ assisted by Glu 106 through Thr 199[16]. (Eq. 8)


Glu 106
Thr 199
Zn
O=C=O
CO2
H2O
OH2

(8)

The protonated imidazole ring of this residue coordinates to zinc with increasing pH, losing a proton. Geometric constraints on the histidine make this a strained and therefore labile metal-ligand bond. However, their work was based on the argument that no data exists to indicate that the pKa of water can be reduced in solution from 15.7 to less than 7 when bound to zinc. Work by several groups subsequently has shown that the pKa of water can be drastically changed in an environment similar to that of the enzyme[30,31].

The catalytic activity of carbonic anhydrase is consistent with a mechanism in which the deprotonated form of an ionizable group on the active site catalyses the hydration of carbon dioxide, while the protonated form catalyses the dehydration of bicarbonate. Consequently turnover of the enzyme requires proton transfer from the active site to the solution in order to regenerate the unprotonated form[32]. (Eq. 10)

$$EH^+ \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E + H^+ \qquad (10)$$

This transformation must occur at a rate greater than or equal to the turnover number ($10^6 s^{-1}$), therefore $k_f \geq 10^6 s^{-1}$. Since the interconversion of the acidic and basic forms of the enzyme involves an ionization of apparent pKa=7, then by Eq. 11:

$$k_r = \frac{k_f}{K_{eq}} = \frac{10^6 s^{-1}}{10^{-7} M} = 10^{13} M^{-1} s^{-1} \qquad (11)$$

This poses a problem since the value of k_r exceeds the diffusion controlled rate constants for proton transfer (10^{10}-$10^{11} M^{-1} s^{-1}$) in water at 25°C[33]. In order to account for the high turnover number it has been proposed that the proton transfer is facilitated by buffers in solution. This hypothesis has been supported by observations of a decrease in the catalytic hydration activity as the concentration of buffers is decreased[34]. These buffers act as proton transfer agents which, when present at large concentrations compared to H_3O^+ or OH^- enhance catalytic activity by donating protons to or accepting protons from the activity controlling group at the active site. It is not known whether this proton transfer occurs directly or through intervening amino acid side chains or water bridges. Recent observations indicate that hemoglobin can provide the required buffer function in vivo[34].

I - E MODEL SYSTEMS

Being proteins, enzymes are extremely complicated molecules and mechanistic interpretation of kinetic data derived from them is generally difficult. A complete description of the mechanism of action of an enzyme requires knowledge of several factors such as the structures

of the active site and of enzyme substrate complexes, the specificity of substrates and their ability to bind to the enzyme and the rate constants and mechanism for every step of the reaction.

The study of small molecule analogues of the metal binding sites in enzymes appears to offer an attractive opportunity to study the metal based chemistry without the protein based complications inherent in studying the enzymes themselves.

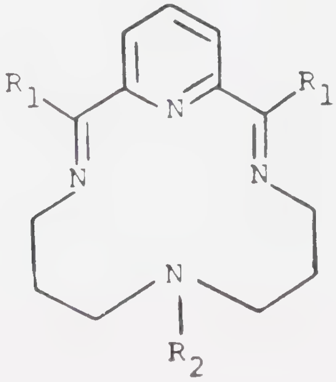
Model work can begin only after enzymologists have provided information about the structure of the enzyme and the identity of the groups in the active site. Once this basic information is available the next step would be to gain understanding of the chemistry of similarly disposed small molecules. It should be stated, however, that the accuracy of the model is only as good as the structural information available[35].

The number of different functional groups that an enzyme can use in the catalytic process is quite limited. Among them are the imidazole ring, aliphatic and phenolic hydroxyl, carboxyl, sulphydryl and amino groups. The problem then is (after assuming the protein structure is not involved) in determining how such functional groups can participate in the reactions and how the rates of the enzymatic reactions can be accounted for in mechanistic terms.

I - F RECENT MODELS FOR CARBONIC ANHYDRASE

Model work on carbonic anhydrase has centered on the design of chelating ligands which approximate the distorted 4(5) coordinate active site or which catalyse certain of the reactions mediated by the enzyme such as CO_2 hydration, aldehyde hydration and ester hydrolysis[31]. As one group has stated: "Any proposed model for the active site structure and the mechanism of action of carbonic anhydrase should take into account those experimental data which are more directly related to the metal ion environment"[36].

One of the recent models for carbonic anhydrase is that of Wooley who has synthesised macrocyclic ligands designed to bind a zinc or cobalt ion in a reduced coordination environment. The complexes of ligands 1 (a-c) are five coordinate with 4 nitrogen donors from the macrocycle and water as a fifth ligand[30].

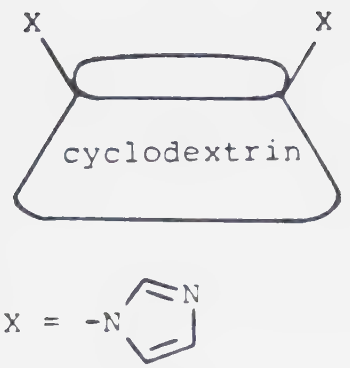


1a R_1=Me, R_2=H
1b R_1=R_2=H
1c R_1=R_2=Me

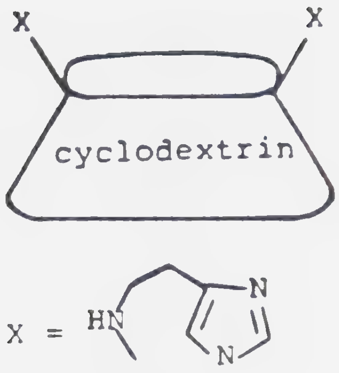
Titration of these metal complexes yielded pKa values of about 8 at 25°C, which has been taken as support

for the presence of the postulated zinc-bound hydroxide ion in carbonic anhydrase at neutral pH values. Since the model ($\underline{1}$:Zn^+:OH) catalyzed acetaldehyde hydration at a rate comparable to that of the enzyme, the metal bound hydroxide has sufficient nucleophilic power to account for the enzyme's activity in acetaldahyde hydration although not in carbon dioxide hydration for which the model provides only modest catalysis. If the same general $Zn^{2+}(OH^-)$ mechanism is to be applied to this latter process then some further enzymatic feature is needed to accelerate the attack on carbon dioxide[37].

Another approach to carbonic anhydrase models was undertaken by Tabushi[38] based on the notion that hydrophobicity is an integral factor in designing catalysts for $CO_2 \rightleftharpoons HCO_3^-$ interconversion. Two models were studied ($\underline{2}$ a-b:Zn^{2+}) in which a cyclodextrin molecule affords the hydrophobic pocket with two pendant imidazoles located at the edge to bind the zinc ion. These compounds proved to be very modest catalysts for CO_2 hydration with k_{cat}=16.2 and 166 $M^{-1}s^{-1}$ respectively while the value reported for human carbonic anhydrase C is $10^5 s^{-1}$.[1e] The second compound affords a larger catalytic enhancement probably because the increased flexibility allows both imidazoles to simultaneously bind to the zinc ion affording a different coordination geometry.



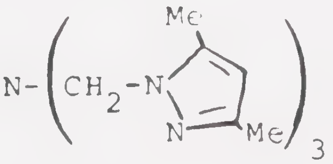
2a



2b

Bertini and co-workers synthesised the complexes $(Co(H_2O)$(tris-(3,5-dimethyl-1-pyrazolylmethyl)amino)) $(ClO_4)_2$ and $(Co(N_3^-)$ (tris-(3,5-dimethyl-1-pyrazolylmethyl) amino))$(Cl_4)_2$ and studied their spectral behaviour[39]. The goal was to investigate the acid-base properties, the influence of water deprotonation on the electronic spectra, and the possibility of substitution of the H_2O or OH^- group by other ligands[40].

The ligand (3) was chosen because pyrazolyl containing ligands are known to stabilize low oxidation states of metals (which should protect Co^{2+}) and allow one water molecule as the fifth ligand. Also pyrazoles were assumed to be similar to imidazoles which are the coordinating groups in carbonic anhydrase[41].

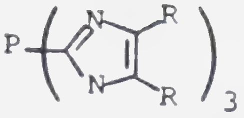


3

The model mimics the spectral variations of the cobalt enzyme in terms of deprotonation of a Co^{2+}-bound water molecule as a function of pH and thus supports those processes that involve bound H_2O and OH^-. However, the model has an extinction coefficient of around 30 while that of the enzyme has a value of 10^2-$10^3 M^{-1} cm^{-1}$ [42], and showed no catalytic activity in carbon dioxide hydration[40].

A quantum mechanical model for carbonic anhydrase has recently been reported[43]. According to this gas phase situation the fourth ligand attached to the zinc is carbon dioxide, and the calculations indicate that direct attack of the hydroxide ion on carbon dioxide is then an almost barrierless process. In solution this reaction has an energy barrier that indicates a different mechanism. In this case the fourth ligand attached to zinc can be a hydroxide ion.

In 1980 Brown and co-workers[44] reported a successful model system exhibiting carbon dioxide hydration catalysis that also approximated the known zinc binding site for carbonic anhydrase. This model consisted of a phosphorous atom surrounded by three imidazole entities substituted in the 4 and 5 positions (4-a,b,c).



4a R=H

4b $R=CH_3$

4c $R=CH(CH_3)_2$

Model 4a formed a 2:1 ligand:metal complex with the metal, so larger sibstituents were then introduced in the 4 and 5 positions to avoid this. Previous work had indicated that by having two bulky substituents (i.e. *iso*propyl groups) at the 4 and 5 positions of the three imidazole rings, the bound metal was sufficiently encapsulated to allow access of only one additional ligand[31].

^1H NMR spectra of 4c in methanol-d_4/D_2O as a function of increasing Zn^{2+} showed the appearance of a well defined 1:1 complex when 4c/$Zn^{2+}=1$; no 2:1 complex was observed. Although U.V. spectra of 4a and 4b in the presence of $CoCl_2$ showed little if any evidence for 4 coordinate ligation, 4c in the presence of $CoCl_2$ showed reversible formation of a tetrahedral species at increasing pH. The 4c:Co^{2+} spectra proved to be highly anion dependent reminiscent of the situation for the Co^{2+}:enzyme[44].

Catalytically 4a and 4b showed negligible activity towards CO_2 hydration, however 4c:Zn^{2+} afforded efficient catalysis for $HCO_3^- \rightleftharpoons CO_2$ interconversion

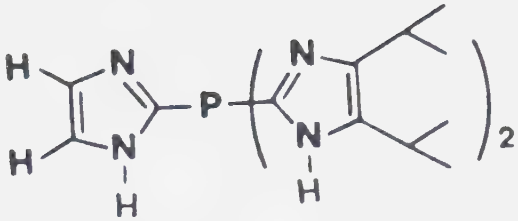
between measured pH's of 6.1 and 7.3[45]. This constituted the first catalytically active model that approximated the binding site for zinc in the enzyme.

A study of the crystal structure of 4c:$ZnCl_2$ revealed that the tridentate coordination to Zn^{2+} rendered a structurally rigid cavity producing severe buttressing for bicarbonate ion binding[20]. This could explain the low catalytic activity observed for the model since the complex would be unable to accommodate the increased coordination required for ligand exchange during the catalytic cycle[45].

Although successful in some aspects, all models studied so far suffer from some problems. The only successful models that show catalytic activity for CO_2 hydration-bicarbonate dehydration are the ones by Tabushi[38] and Brown[44]. The former suffers from the fact that only two imidazoles are bound to the metal in the case of 2b and the role of the additional bases is debatable, while the structure of 2a does not allow both imidazoles to coordinate to the metal. Thus little can be said about the mode of action of the model catalyst compared to the enzyme. The latter (4c:Zn^{2+}) does successfully mimic a number of physicochemical features of the active site of the enzyme, but the activity is too low, presumably due to the restrictive coordination about the Zn^{2+}.

The object of the present work was to synthesise and to study other phosphine imidazole ligands as models for carbonic anhydrase. Compounds 5,6, and 7 were designed to provide a Zn^{2+}-binding cavity with greater accessibility to the reagents and at the same time enforce low co-ordination at the metal. This was done by substituting the bulky *iso*propyl groups in one of the imidazoles with a less restrictive hydroxyethyl group or hydrogen. Ligand 6 contains the remote hydroxyethyl group which was introduced to mimic the possible interaction of the active site threonine group which has been postulated to assist during the hydration of CO_2 by the enzyme. For this model an additional complication must be considered since the 4(5)-hydroxyethyl group renders the two nitrogens inequivalent and therefore provides the possibility that non-ideal binding to the metal would locate the OH group in too distant a position to provide any catalytic enhancement. Ligand 7 was designed with the additional advantage of providing a more hydrophobic environment for the catalytic process. This ligand was kindly provided by Dr. Henrika Śleboka-Tilk.

The following represents the findings during the course of this study.



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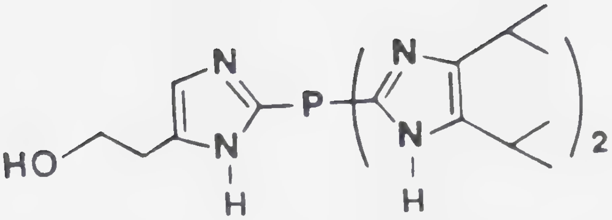

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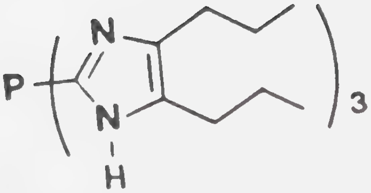

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CHAPTER II

RESULTS AND DISCUSSION

II - A SYNTHESES

The three ligands studied were made using the general method outlined by Brown and Curtis[46]. This consisted of initial protection of the imidazole nitrogen by formation of its N-(dimethoxymethyl) or N-(diethoxymethyl)-acetal. The original procedure reported for protecting 4,5-di*iso*propylimidazole involved refluxing a toluene suspension of trimethyl orthoformate and the imidazole in the presence of *p*-toluenesulfonic acid and removing methanol as it was formed. However, this synthesis proved to be irreproducible and only small yields of the desired material could be isolated. Further investigation indicated that formic acid, (which was present as an impurity in the original trimethyl orthoformate used) proved to be the essential catalyst. Yields in excess of 75% can routinely be obtained on a 0.1 M scale if 2 ml of formic acid are added to the reaction mixture. Protection of the other imidazoles did not present any further complications.

Once the imidazoles were protected, their lithium salts were prepared and these were used to perform a nucleophilic displacement on phosphorous trichloride.

The desired phosphines were obtained by deprotection of the imidazole and purification of the resulting mixture. Scheme 1 depicts the pathways followed for the construction of the ligands. The resulting phosphines were recrystalyzed from methanol/water giving overall yields of 20-30% based on starting material.

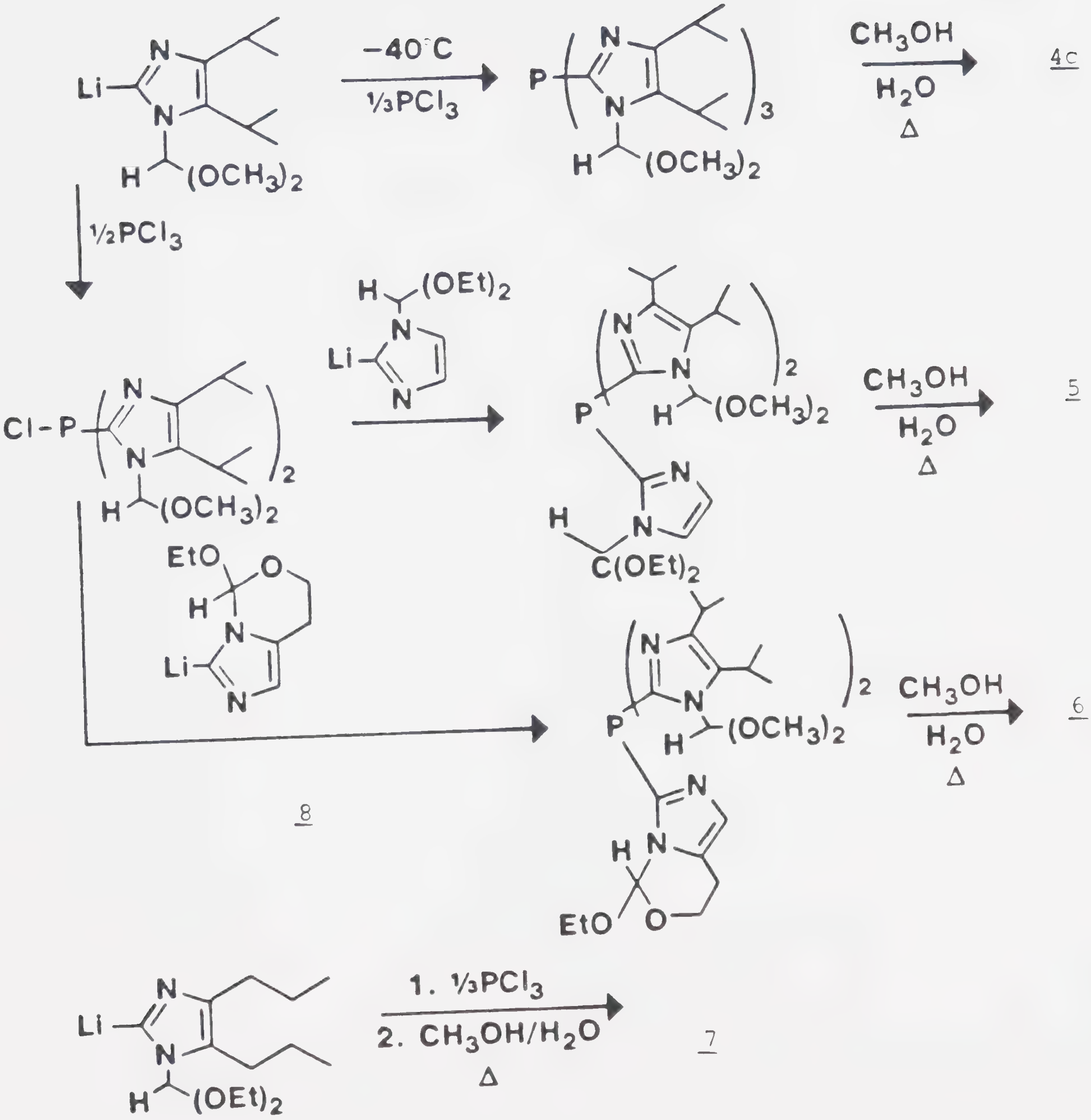
II - B H^+ AND M^{2+} BINDING CONSTANTS

In order to be considered good models for the active site of carbonic anhydrase the ligands must be shown to bind Zn^{2+} (or Co^{2+}) strongly in a tridentate fashion. Ionization constants of the protonated ligands were determined by potentiometric titrations, the data being analyzed by a computer version of the Simm's method[47]. The metal binding constants (pK_M2+) were also determined by potentiometric titrations and the data analyzed with the aid of a computer[48]. Table 1 lists the ligand pKa and $pK_M{}^{2+}$ values under two different sets of conditions.

Due to solubility problems the experiments were carried out in a medium consisting of 80% ethanol and 20% water (V/V). All of the ligands studied showed a greater propensity for binding zinc than cobalt, a property they share with native carbonic anhydrase.

Quantitative titration experiments on solutions consisting of equimolar Zn^{2+} and ligand with 2 equivalents of added HNO_3 showed that all of the proton is accounted

Scheme 1


Li
N
N
H
(OCH$_3$)$_2$
−40°C
⅓PCl$_3$
P
3
CH$_3$OH
H$_2$O
Δ
4c
½PCl$_3$
Cl−P
2
(OEt)$_2$
5
EtO
O
C(OEt)$_2$
6
8
1. ⅓PCl$_3$
2. CH$_3$OH/H$_2$O
7

for at a pH lower than the third pKa of the ligand (i.e. by pH 5 for ligand 5) which indicates that a complex is fully formed by these pH's. After all of the added proton has been accounted for there appears to be an additional ionization at somewhat higher pH but precipitation occurs before any well defined pKa can be obtained. In order to determine the pKa of this new dissociation, an attempt to titrate the ligand-metal complex of 5:Zn^{2+} without added acid failed as precipitation occured by pH 7 in the case of the Zn^{2+} complex and pH 8.3 in the case of Co^{2+}. The expectation was to identify an ionizing group (Zn^{2+}-OH_2) which could be related to the activity controlling group in the enzyme (which most researchers believe to be a metal-bound water molecule). Although the pKa of this ionizing group in the model compounds could not be determined, it should be mentioned that in titrating the cobalt complex of 5, a slight blue color indicative of tetra or pentacoordinate cobalt coordination is observed at a pH of 8.0 thus affording some evidence for low coordination around the metal ion.

Titration was performed employing two different solutions of NaOH since under the first set of conditions ($NaOH_{(aq)}$) ionic strength and solvent composition were constantly changing.

TABLE 1

LIGAND[a]	pKa_1[b,c,e]	pKa_2[b,c,e]	pKa_3[b,c,e]	$pK_{Zn^{2+}}$[b,d,e]	$pK_{Co^{2+}}$[b,d,e]
5	<1.8 *	4.1	6.9	9.0	7.1
6	<0.5 *	3.7	6.7	7.5	7.1
7	2.2	4.4	6.9	9.1	7.1

a) As defined on p.19

b) Values reported are averages of at least three determinations and have a ± 0.1 unit precision.

c) For $L + H^+ \rightleftharpoons LH^+$ $\quad Ka = \frac{[L][H^+]}{[LH^+]}$

d) For $M^{2+} + L \rightleftharpoons ML^{2+}$ $\quad K_M{}^{2+} = \frac{[M^{2+}][L]}{[ML^{2+}]}$

e) Titration performed with 0.1 N $NaOH_{(aq)}$ as described in Experimental section.

* value not directly determined but estimated

TABLE 1 (Continued)

LIGAND[a]	pKa_1[b,c,e]	pKa_2[b,c,e]	pKa_3[b,c,e]	$pK_{Zn^{2+}}$[b,c,e]	$pK_{Co^{2+}}$[b,c,e]
5	<1.8*	3.5	6.6	8.7	7.68
6	<0.5*	3.5	6.2	6.54	5.76

a) As defined on p. 19

b) Values reported are averages of at least three determinations and have a ± 0.1 unit precision.

c) For $L + H \rightleftharpoons LH^+$ $\quad Ka = \frac{[L][H^+]}{[LH^+]}$

d) For $M^{2+} + L \rightleftharpoons ML^{2+}$ $\quad K_M{}^{2+} = \frac{[M^{2+}][L]}{[ML^{2+}]}$

e) Titration performed with 0.2 N NaOH in 80% EtOH and 20% water (v/v) as described in Experimental section.

* value not directly determined but estimated

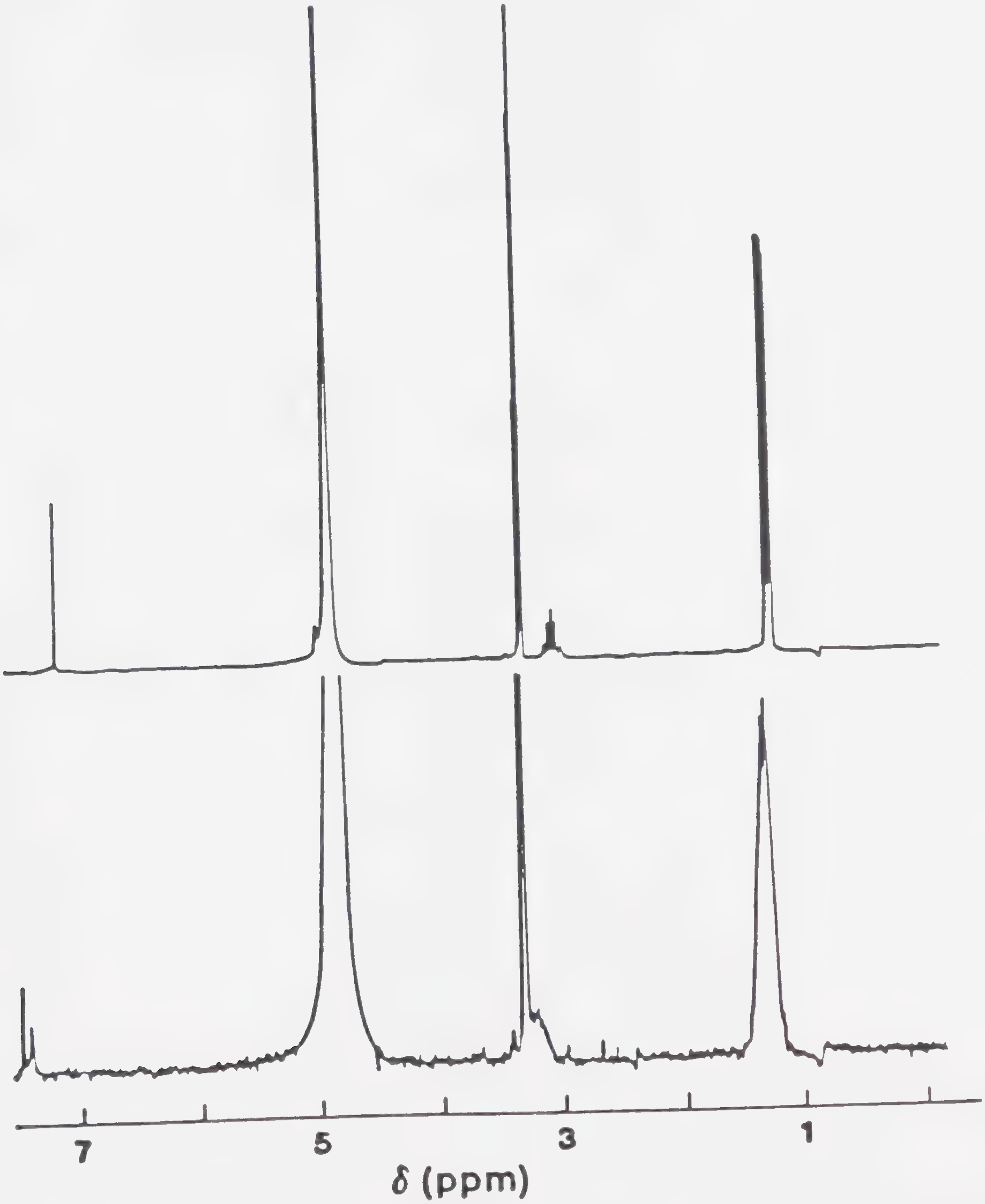
II - C NUCLEAR MAGNETIC RESONANCE STUDY FOR Zn^{2+} BINDING

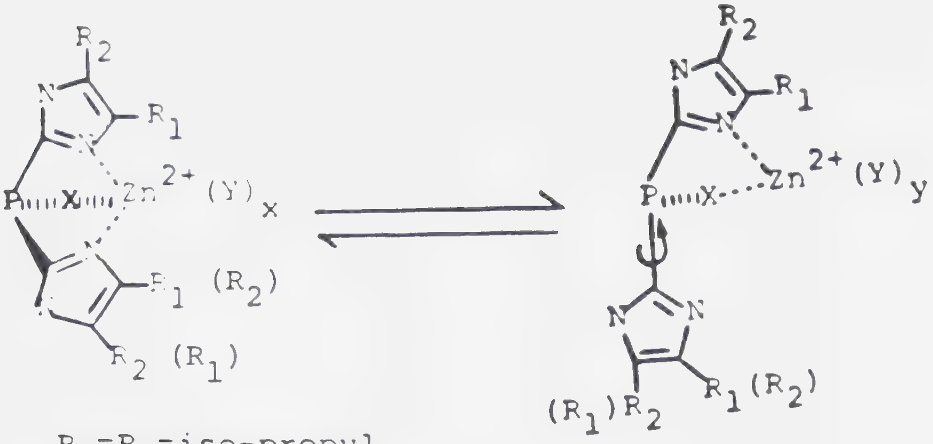
^1H NMR analyses were undertaken on 0.02M solutions of each of the three ligands in CD_3OD as a function of added aliquots of $ZnCl_2$ and $Zn(ClO_4)_2$ in D_2O. These experiments were performed in order to determine whether the complex formed between ligand and metal was a rigid tridentate complex or if some dynamic exchange (slow enough to be detected on the NMR timescale) was occuring and to assay the effect of the ligand/metal ratio on the spectrum of the complex.

Figure 1 shows the results obtained for ligand 5 with added $ZnCl_2$. When the ligand to zinc ratio is one, the original *iso*propyl signals have been replaced with broadened resonances centered at 1.3 ppm. The *iso*propyl methine signals have also broadened and moved downfield while the imidazole 4 and 5 hydrogens appear as separate signals at 7.3 and 7.4 ppm. Further addition of zinc produces no additional changes indicating that the compound exists as a 1:1 complex experiencing a dynamic exchange process involving the *iso*propyl imidazoles and the zinc ion perhaps as in eq. 12.

FIGURE 1

^{1}H NMR spectrum of 5 as a function of added $ZnCl_2$. Resonances centered at δ4.8 and 3.3 are from HOD and deuteriomethanol solvents.





(12)

R_1=R_2=iso-propyl

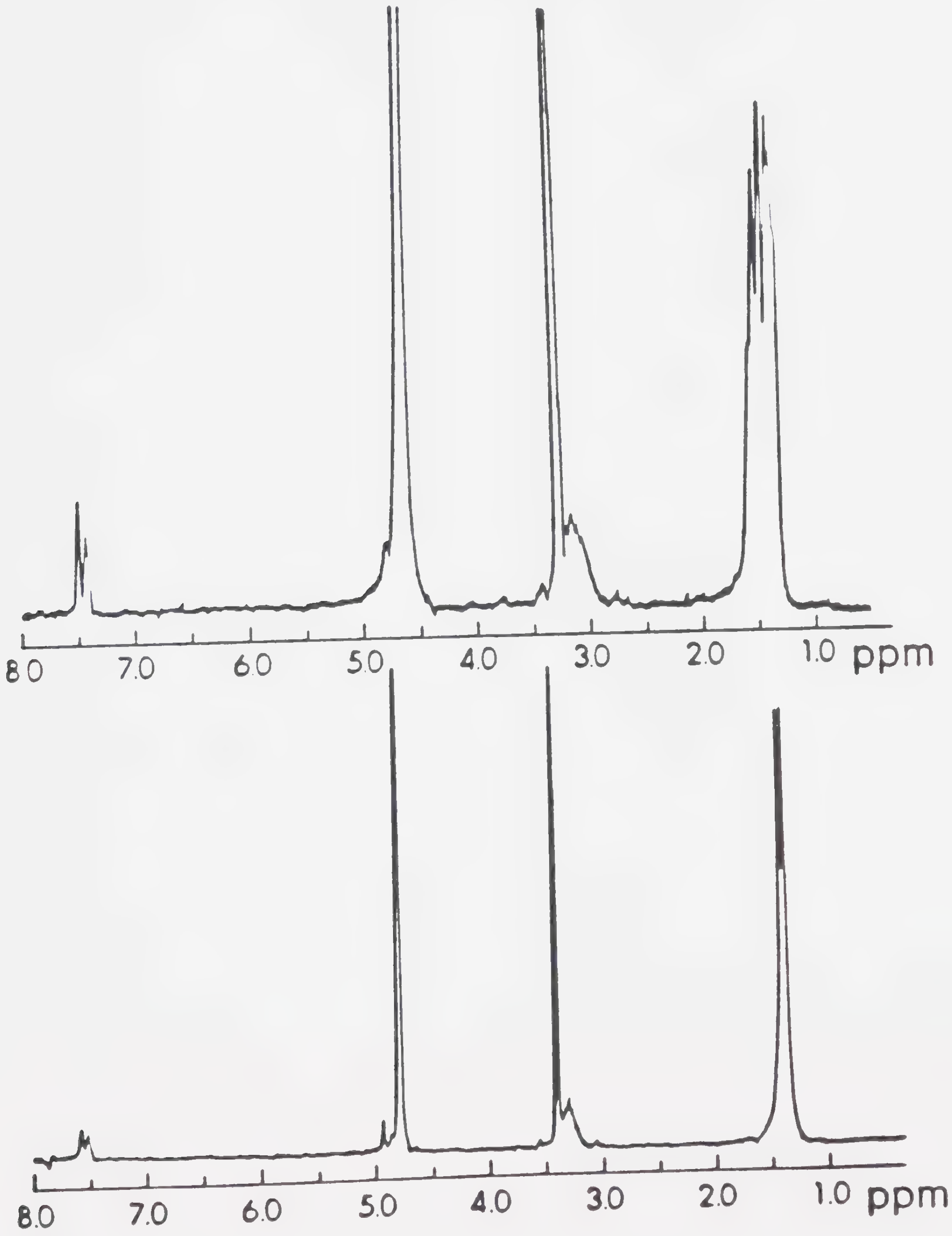
X = imidazole

A similar experiment conducted using aliquots of $Zn(ClO_4)_2$ gave identical results.

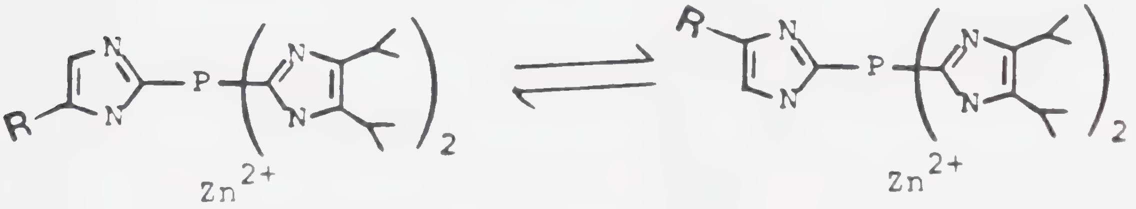
By obtaining the NMR spectra of ligand 5 in a 1:1 ratio with $ZnCl_2$ at two different temperatures, the proposal of a fast site exchange of the diisopropyl imidazoles seems to be confirmed. Figure 2 shows the spectra at two different temperatures. The lower temperature spectrum (0°C) shows a different splitting pattern for the isopropyl groups indicating that, on the average, they experience different environments which is expected if at low temperature the exchange rate is slowed down. The high temperature (50°C) spectrum shows that the isopropyl groups have been brought into a more equivalent environment since the exchange rate is fast on the NMR timescale.

FIGURE 2

^{1}H NMR spectrum of $\underline{5}$:Zn^{2+} as a function of temperature. Top spectra t=0°C; bottom spectra t=50°C.



Ligand 6 gives similar results. Figure 3 shows that when the Zn^{2+}:ligand ratio is 1:1, the diisopropyl imidazole signals broaden out, the methyl signals are centered around 1.2 ppm and the methine ones around 3.2 ppm (overlapping with the methanol). The hydroxyethyl imidazole signals are comprised of a triplet at 3.8 ppm attributable to the methylene unit closest to the oxygen atom while two distinct signals at 2.9 and 3.1 ppm. can be ascribed to the other methylene unit in two environments. These broadened triplets indicate two different forms of the complex in slow equilibrium (Eq. 13). The fact that the 4(5)-hydrogen appears as two distinct singlets at 7.2 and 7.4 ppm tends to support this idea.



(13)

$R = CH_2CH_2OH$

Using equimolar $Zn(ClO_4)_2$ instead of $ZnCl_2$ gives results that are quite different. The methylene units appear as very broad signals while the 4(5)-hydrogen appears as a broad singlet. Addition of more Zn^{2+} shows no appreciable changes, so that the spectrum is indicative

FIGURE 3

^{1}H NMR spectrum of 6 as a function of added $ZnCl_2$.

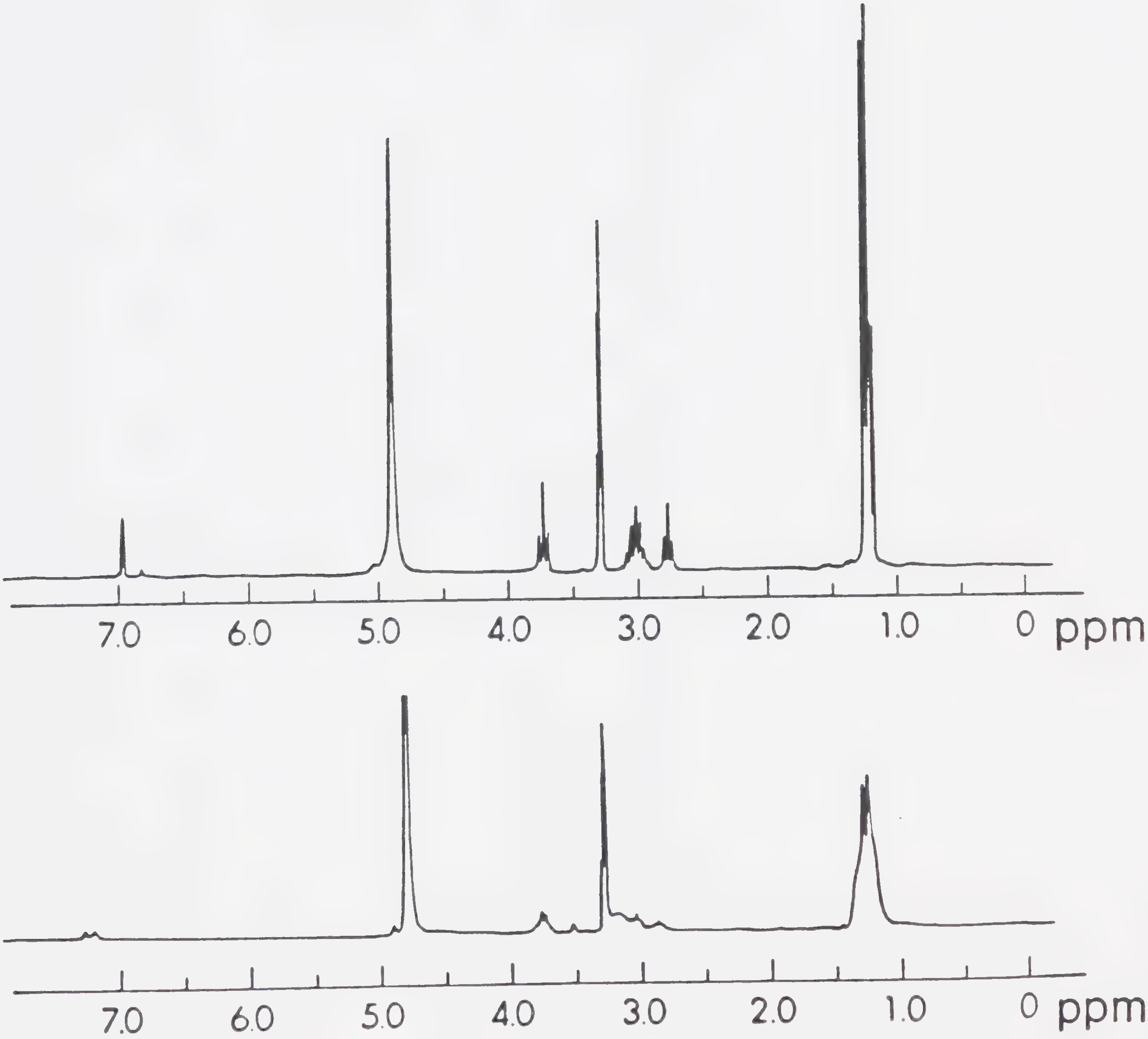
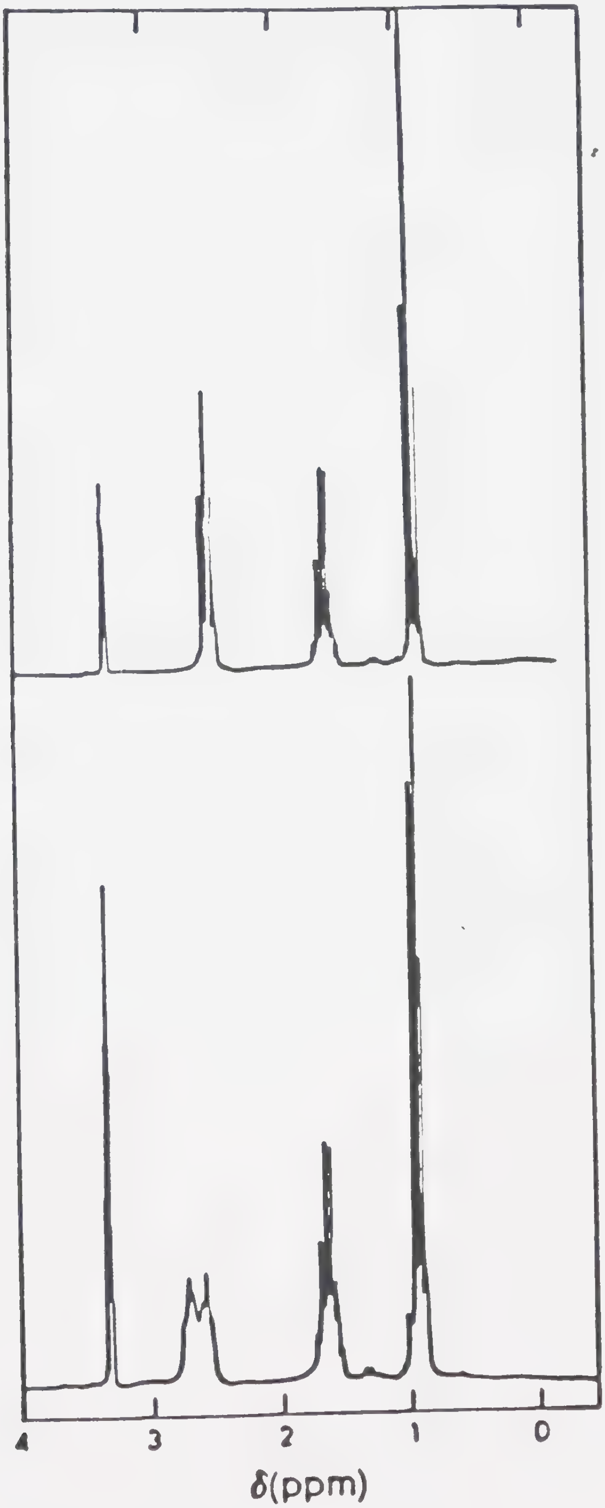


FIGURE 4

^1H NMR spectrum of 7 as a function of added $ZnCl_2$



of a 1:1 complex. This tends to indicate that the nature of the complex and its dynamic exchange properties are dependent on the counterion.

The situation for ligand 7 is quite different as is shown in figure 4. As in the previous two cases a 1:1 complex is formed and further addition of $ZnCl_2$ shows no appreciable change. However, for this ligand the methylene units next to the 4 and 5 positions are clearly split into two overlapping triplets of equal intensity at 2.6 and 2.75 ppm. This is expected if tridentate complexation occurs and dynamic exchange on the NMR timescale is slow. The rigid tridentate complex that forms is, as before, dependent on the counterion and when $Zn(ClO_4)_2$ is used the NMR spectrum of the 1:1 complex is indicative of a complex undergoing dynamic exchange which brings the 4 and 5 CH_2's resonances into a more averaged environment.

II - D Co (II) ABSORPTION SPECTRA

The fact that substitution of Co(II) for Zn(II) in native carbonic anhydrase gives an enzyme that is partly active provides the possibility of using the spectroscopic properties of cobalt as a probe for the active site geometry of carbonic anhydrase.

The Co^{2+} ion has a d^7 configuration and preferentially forms octahedral complexes although four and five coordination is not uncommon[49]. The d-d absorption spectra of Co(II) are so characteristic that octahedral and tetra or pentacoordinate geometries complexes can be differentiated[48]. Generally, octahedral Co(II) is pink (λ max. around 500 nm, ε around 10 $M^{-1}cm^{-1}$), whereas tetrahedral cobalt is blue or violet (λ max. $\geq$ 575 nm, ε about 200-1000 $M^{-1}cm^{-1}$). Pentacoordinate Co(II) complexes have absorption bands between 500 and 700 nm with intensities intermediate between those of the tetrahedral and octahedral species.

In order to compare the spectroscopic properties of the ligands:Co^{2+} under consideration with those of the Co(II) enzyme, equimolar amounts of $CoCl_2$ and ligand were combined and the UV-visible spectrum recorded as a function of pH. For ligand 5 this showed little evidence of formation of a 4 coordinate complex. The ε max in the 600 nm region was found to be about 10 $M^{-1}cm^{-1}$ rather than the expected[42] 10^2-10^3 $M^{-1}cm^{-1}$ (figure 5). Ligand 6 behaved in a very similar manner providing very little evidence for a tetrahedral or penta-coordinate complex. However, ligand 7 shows a definite build up of a tetrahedral complex between pH 2 and 4.5 (ε max = 720 $M^{-1}cm^{-1}$,

FIGURE 5

Visible absorption spectra of 5:Co(II) 8.3×10^{-3} M; 0.2 M NaCl) as a function of pH. Solvent 80% EtOH:H_2O. Temp=25.0 ± 0.2°C

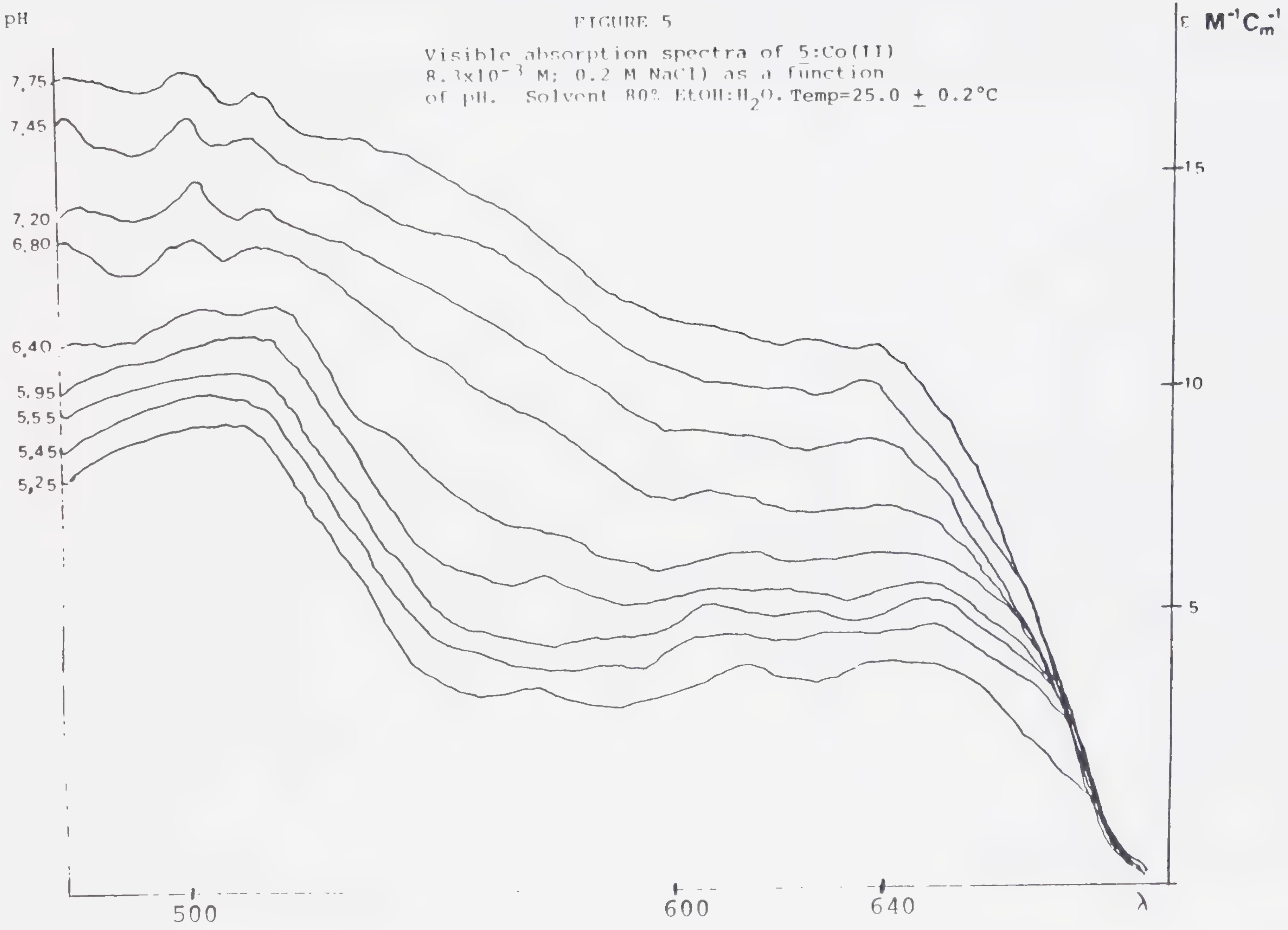


FIGURE 6

Visible absorption spectra of 7:Co(II) (7.5×10^{-4} M; 0.2 M NaCl as a function of pH. Left hand spectra increase in intensity from pH 2 → 4.5; right hand spectra decrease in intensity from 4.5 → 8. Solvent 80% EtOH:H_2O.

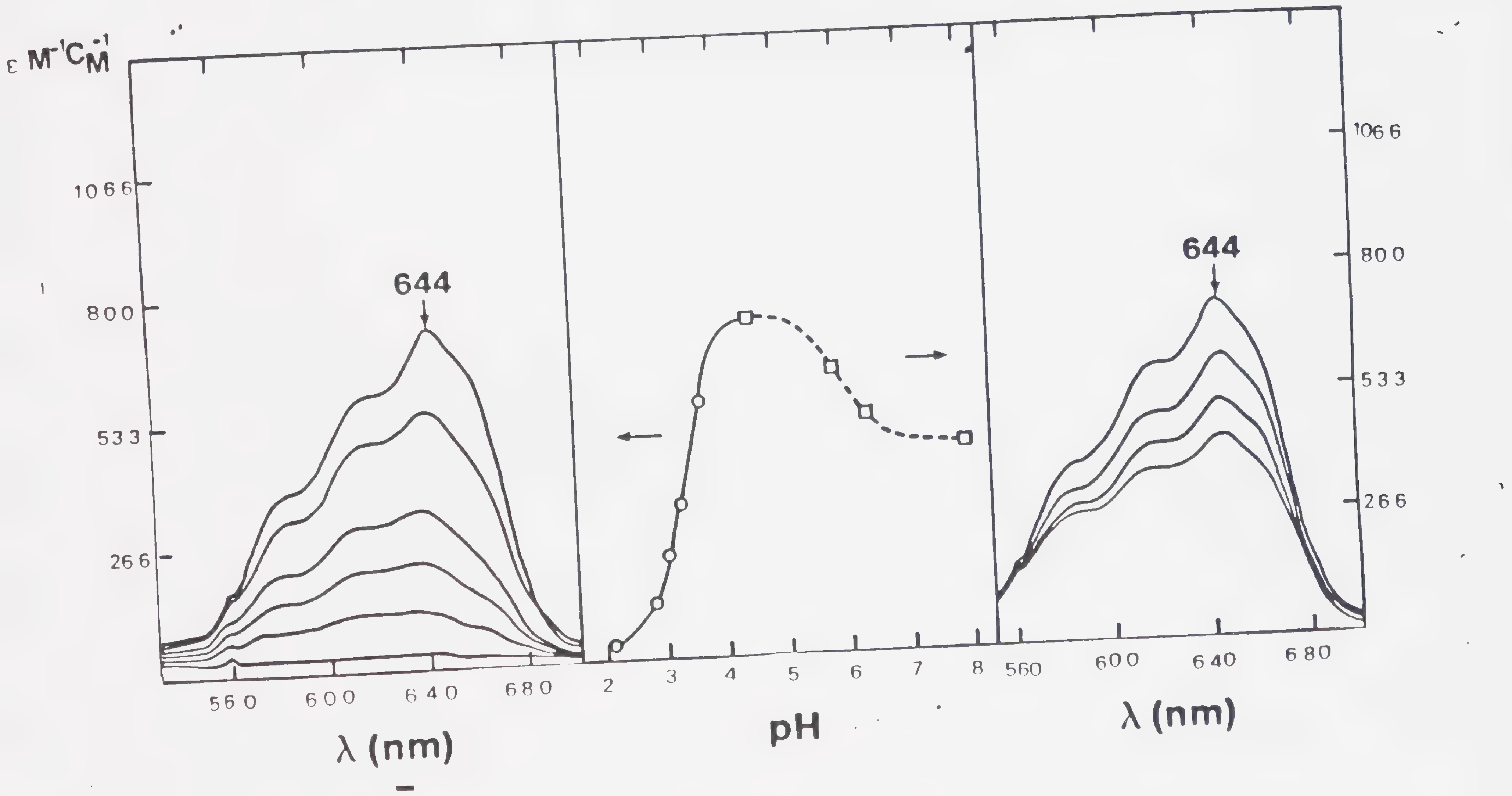
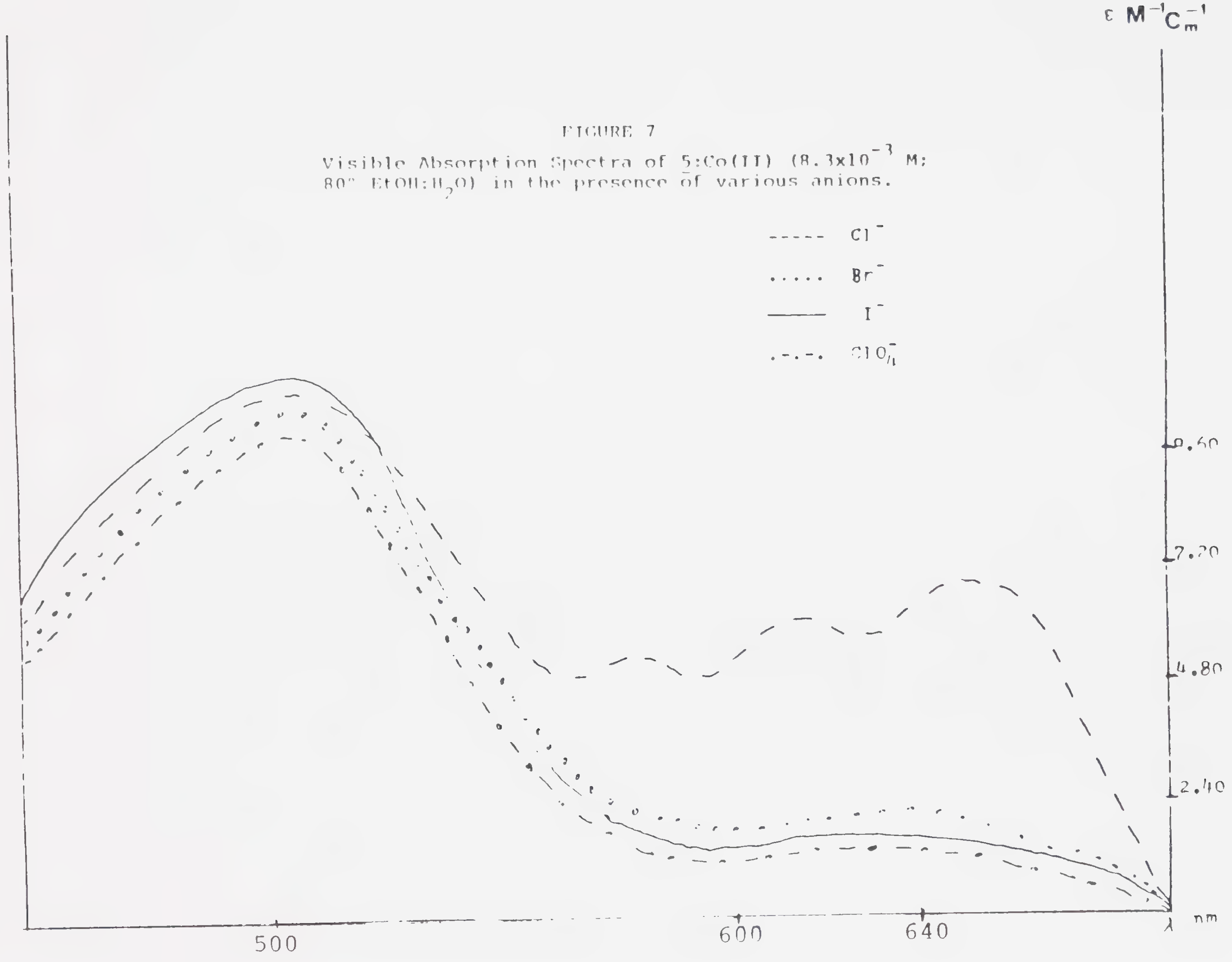


FIGURE 7

Visible Absorption Spectra of 5:Co(II) (8.3×10^{-3} M; 80% EtOH:H_2O) in the presence of various anions.



λ 644 nm. at pH 4.5). Above pH 4.5, the intensity of the 644 nm band diminishes and the shape changes which suggests formation of a second 4 or 5 coordinate complex. (figure 6). Quantitative measurement of the consumption of OH^- suggests that hydroxide is consumed during the course of the transformation by association with the Co(II) or by titration of a metal bound water and from figure 6 a spectroscopic pKa of around 6 can be determined. Precipitation occurs at pH>8. These observations are accommodated in Scheme 2.

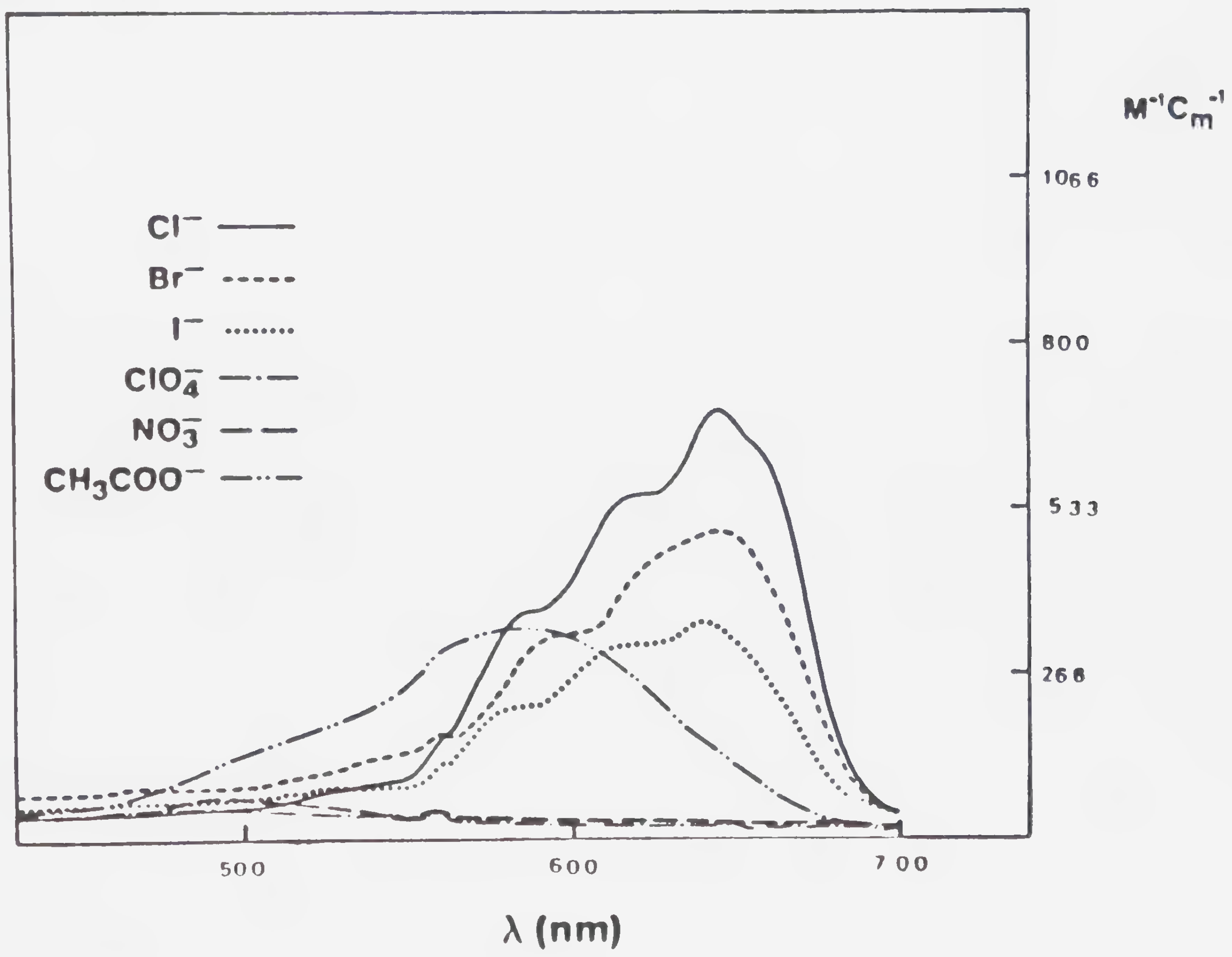
The dependence of the Co(II) spectra upon associated anions at pH 6.4 was also studied. Figure 7 shows the UV-visible spectra of 5 in the presence of added anions. It can be seen that the maximum absorption at around 600 nm occurs when the anion is chloride, however, the value of ε max is still small and therefore most of the complex exists in an octahedral form. Complexes of 6 display the same type of behaviour towards different anions, however, those of 7 have a higher extinction coefficient indicating that there is a considerable amount of tetrahedral complex.(figure 8). One can establish the following order for decreasing absorption at 600 nm:

$$Cl^- > Br^- > I^- > ClO_4^-$$

A control experiment was run with different anions and no ligand, the only absorption observed was at 500-

FIGURE 8

Visible absorption spectra of 7:Co(II) (7.5×10^{-4} M; 80% EtOH:H_2O) in the presence of various anions.

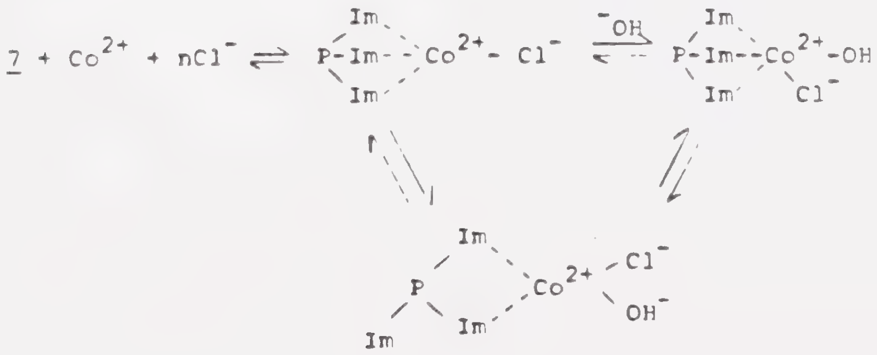


510 nm and all anions gave the same spectrum. This indicates that the absorption at around 600 nm is due to a complexation between ligand, metal and anion.

The Co(II) spectrum with bicarbonate anion could not be determined due to precipitation problems. Therefore it is not possible to conclude if some of the complex formed between ligand and bicarbonate anion exists in a tetrahedral form.

From the above experiments it can be concluded that there is a strong dependence of the Co(II) visible spectra of the ligands under study on the presence of associated anions. Ligand 7 tends towards tetrahedral coordination when the associated anions are halides thus indicating that low coordinate 7:Co(II) complexes require an associated anion in at least one of the available metal sites. Ligand 5 shows a slight tendency to adopt a low coordinate complex structure but the absorbance in the 600 nm region is still very low so that most of the complexes in solution must be octahedral.

SCHEME 2



II - E CATALYTIC STUDIES

The catalytic activity of the Zn^{2+} complexes of 5, 6, and 7 towards bicarbonate dehydration was studied employing indicator techniques[28,50] in which the change in $[H^+]$ accompanying the reaction is monitored by observing an absorbance change of an indicator anion (A^-) whose response to $\Delta[H^+]$ is rapid compared to the reaction under study. (Eq. 14).

$$AH + HCO_3^- \xrightleftharpoons{\text{fast}} A^- + H^+ + HCO_3^- \xrightleftharpoons[k_r]{k_f\,(\text{slow})} H_2O + CO_2 + A^- \qquad (14)$$

For the present work HEPES buffer held $[H^+]$ essentially constant so that the reactions were run under pseudo-first order conditions. $[H^+]$ must vary to some minor extent since it provides the observable for the kinetic analysis but under the appropriate buffering conditions $\Delta[H^+]$ can be held to less than 0.04 pH unit (8%). Under these conditions Eq. 14 can be reduced to a simple first order equilibrium situation $HCO_3^- \xrightleftharpoons[k_r]{k_f} CO_2$ and therefore k_{obs} is equivalent to $(k_f + k_r)$, the sum of all the forward and reverse rate constants which includes those dependent upon the various forms of the buffer and other species present in the medium[51]. The rate of change in $[HCO_3^-]$ can then be given by Eq. 15:

$$\frac{-d[HCO_3^-]}{dt} = \frac{d[CO_2]}{dt} = \frac{d[A^-]}{dt} = k_{obs}[X_e - X] = (k_f + k_r)\,[X_e - X] \qquad (15)$$

where A^- indicates the concentration of indicator anion, X and X_e denote the concentration of the product of the reaction at time t and equilibrium respectively, and k_{obs} is a measure of the rapidity with which equilibrium is attained.

Of the three ligands studied 5 was expected to provide a larger catalytic activity than the previously studied 4c since the former provides a more accessible cavity into which bicarbonate should be able to fit without the severe buttressing anticipated for 4c. Ligand 6 was expected to mimic the possible interaction of the active site threonine OH and, if this were an important requisite for the mechanism of action, increase the observed rates considerably. Ligand 7 was designed to provide not only a more accessible cavity but also a more hydrophobic environment for increased catalysis. The three compounds under study proved to be catalytically active, however, 6 showed only a very small activity. Table 2 shows the results obtained for k_{obs} for the catalysed bicarbonate dehydration at different pH's.

In an 80% EtOH/H_2O medium, the attainment of equilibrium for bicarbonate dehydration is a relatively rapid process having an uncatalysed half time of 0.5 to 1.7 sec. between pH 6.05 and 6.85 respectively. Figure 9 shows a plot of k_{obs} as defined in equation 15 vs. pH.

TABLE 2

k_{obs} Values for Bicarbonate Dehydration at Different pH's[a,b]

pH^c	$k_{obs}(s^{-1})^d$				$k_{cat}^{obs}(M^{-1}s^{-1})^e$		
	No ligand	$\underline{5}:Zn^{2+}$	$\underline{6}:Zn^{2+}$	$\underline{7}:Zn^{2+}$	$\underline{5}:Zn^{2+}$	$\underline{6}:Zn^{2+}$	$\underline{7}:Zn^{2+}$
6.2	1.13	1.13	1.17	1.68	0	0	2200
6.3	0.97	1.10	--	1.53	520	--	2240
6.45	0.80	1.08	0.85	1.45	920	50	2400
6.55	0.65	1.03	--	1.27	1520	--	2480
6.70	0.45	0.75	0.51	1.02	1200	60	2260
6.80	0.42	0.58	--	0.86	640	--	1760

---

a.) 80% EtOH/H_2O; 25.0 ± 0.2°C; $1x10^{-3}$ M $NaHCO_3$; $2.5x10^{-2}$ M HEPES; ionic strength 0.2 M ($NaClO_4$); Catalyst = 2.5 x 10^{-4}M when present.

b.) Followed by monitoring the appearance of bromocresol purple anion at 580 nm, BCP = 5 x 10^{-4}M.

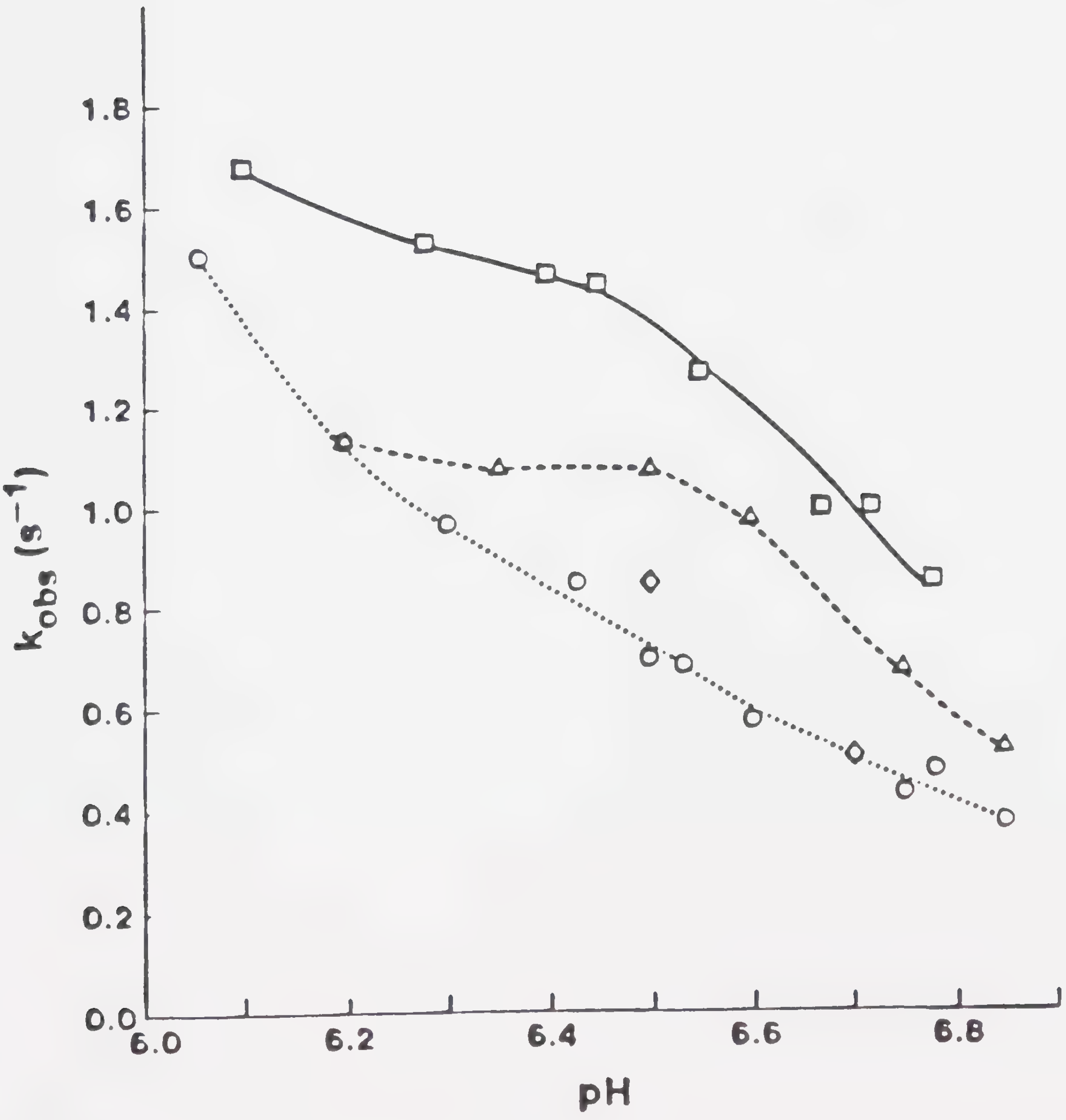
c.) pH read directly from pH meter with no corrections made for the high organic content of the solvent.

d.) Values of k_{obs} ± 0.03 units.

e.) $k_{cat}^{obs} = (k_{obs}(\text{with catalyst}) - k_{obs}(\text{no catalyst}))/[\text{Catalyst}]$

FIGURE 9

k_{obs} as a function of pH for $HCO_3^- \rightleftharpoons CO_2$ equilibration approached from HCO_3^- dehydration. ⊙ - no catalyst; △ 2.5×10^{-4} M 5:Zn^{2+}; ⊡ - 2.5×10^{-4} M 7:Zn^{2+}; ◇ - 2.5×10^{-4} M 6:Zn^{2+}. 80% EtOH:H_2O; 0.2 M $NaClO_4$; 5×10^{-4}M bromocresol purple indicator; 1×10^{-3} in $NaHCO_3$; 2.5×10^{-2} M HEPES



It can be seen that the largest difference between the blank and catalysed reactions occurs with 7:Zn^{++}, followed by 5:Zn^{++}, the difference being maximal at pH 6.4 - 6.5. As in the case of a previously reported catalyst (4c)[45], the activities of these models are maximal at pH 6.4 - 6.5 and are nearly gone at pH <6 and >7. The reason for this has been ascribed to protonation of the ligand at low pH and OH^- sequestering of the Zn^{2+} from the complex at high pH, both processes leading to an inactive species[31b].

Control experiments were performed by monitoring the reaction with added Zn^{2+} but no ligand and vice versa. In both cases the values obtained for k_{obs} were the same as for the uncatalysed reaction so that at these concentrations neither Zn^{2+} nor ligand alone facilitates the reaction. Changing the concentration of Zn^{2+} in a ligand containing solution from 1 to 2 equivalents produced no further change in the value of k_{obs}. These results tend to support the idea that the catalytically active species is a 1:1 complex between ligand and zinc and that the complex is fully formed under these conditions.

Other control experiments were performed in order to test the expectation that the proton transfer step in the indicator is fast. These consisted of monitoring the changes in absorbance at different wavelengths after

rapid mixing of solutions of ligand-metal complex and indicator in the absence of bicarbonate. Within the timescale of the experiment (<20 msec.), only a rapid dilution effect was seen thus indicating that the indicator acid-base reaction is rapid relative to $HCO_3^- \rightleftharpoons CO_2$ interconversion.

The possibility of having a dependence of k_{obs} on indicator concentration was tested by monitoring the reaction with different concentrations of indicator. A similar control experiment had previously been made for 4c:Zn^{2+} in which the indicator concentration was increased from 5×10^{-5} to 2×10^{-4} M with no change in k_{obs}. For 5:Zn^{2+} the situation is the same since changing the indicator concentration by a factor of 2 produced no change in k_{obs}. However, 7:Zn^{2+} showed a definite dependence at low indicator concentrations since k_{obs} increased as a function of indicator concentration up to a value of 2.5×10^{-4} M after which k_{obs} became independent of indicator concentration. A linear dependence on [ligand:Zn^{2+}] was observed throughout. The solubility of 7:Zn^{2+} depends upon indicator concentration and at low concentrations of the latter (2×10^{-4}M) precipitation was observed. This leads to the conculsion that 7:Zn^{2+}, (and probably the other ligands as well,) forms a complex with the indicator the nature of which is unknown. Thus the possibility of the

catalytic species being a complex of ligand-metal-indicator cannot be ruled out. Unfortunately it is impossible to perform a control experiment without the indicator since there would be no observable property to follow. Attempts were made using other indicators but these were fruitless due to solubility reasons or to the fact that the acid-base indicator ranges were outside the accessible pH range in this highly organic medium.

Since carbonic anhydrase activity seems to depend on the concentration of buffers for its high turnover number[34], an experiment was performed with 5:Zn^{2+} decreasing the buffer concentration by a factor of 2. This produced no appreciable change in the values of k_{obs}. Unfortunately further changes could not be studied since these led to large variations of pH during the kinetic runs and therefore did not give meaningful results for k_{obs}.

Since substitution of Co^{2+} for Zn^{2+} in the native enzyme leads to an enzyme that retains some of its activity, an accurate model might be expected to show both Zn^{2+} and Co^{2+} activity. It was found that the 5:Co^{2+} complex does show catalytic behaviour and shows about 60% of the activity of the Zn^{2+} complex. By taking advantage of the fact a low coordinate 5:Co^{2+} complex should, if formed be detected spectroscopically at

~600 nm, an experiment was performed by stopped flow mixing a solution of 5:Co^{2+} with another containing HCO_3^- to try to detect transient formation of a tetra or penta coordinated bicarbonate-metal-ligand complex. However, this could not be detected under these conditions so that no conclusion could be made from this experiment.

The fact that all of the experiments are run in a highly organic medium (80% EtOH/H_2O) led to a study on the effects of solvent composition on the rate of the reaction since this could be partly responsible for the catalysis observed. Ligand 5 proved suitable for this purpose since it would dissolve in solutions of varying ethanol content (60,70,80 and 90% EtOH/H_2O). The results of the experiment are presented in Table 3 and indicate k_{obs} increases from 60% to 80% EtOH. Control experiments were performed on solutions that contained no ligand or metal and differed only in alcohol content. No change in k_{obs} was observed in these solutions. Therefore a more alcoholic solvent apparently increases the catalytic effect of the complex.

The effect of catalyst concentration on k_{obs} was assayed by measuring k_{obs} at the optimal pH for the ligands as a function of different complex concentrations. Table 4 shows the values obtained for ligand 5 at pH 6.45 and from figure 10 it can be seen that a linear relationship exists between catalyst concentration and

TABLE 3

Effects of Solvent Composition on k_{obs}[a,b]

% EtOH	k_{obs} s^{-1}	k_{cat}^{obs} $M^{-1}s^{-1}$[d]
60	0.68	258
70	0.90	896
80	1.08	1549
90	1.13	1734

a.) 25 ± 0.2°C; $1x10^{-3}M$ $NaHCO_3$; $2.5x10^{-2}M$ HEPES; ionic strength 0.2 M ($NaClO_4$); Catalyst ($\underline{5}$:Zn^{2+}) = $2.5x10^{-4}M$ when present.

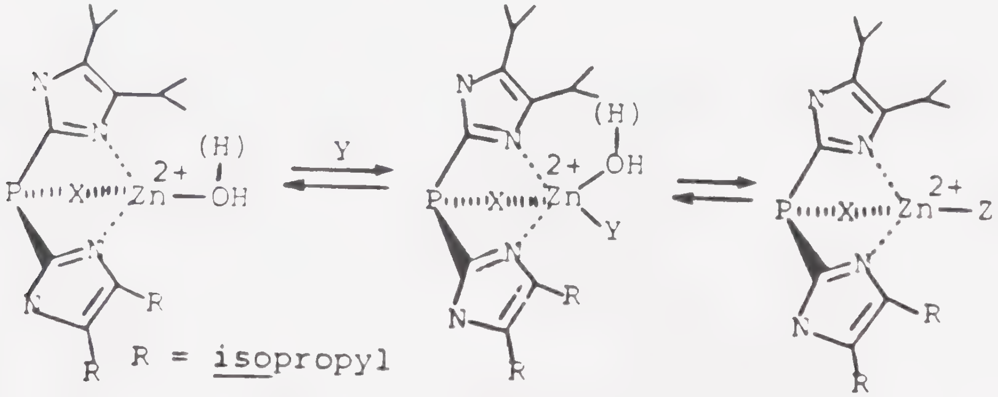
b.) Followed by monitoring the appearance of bromocresolpurple anion at 580 nm, BCP = $5x10^{-4}M$.

c.) Values for k_{obs} ± 0.03 units.

d.) k_{cat}^{obs} = (k_{obs}(with catalyst)-k_{obs}(no catalyst))/[Catalyst].

k_{obs} for ligands 5 and 7. A third ligand, 4c, which has been previously reported[45] is included for comparison purposes. From the slopes of the lines in Figure 10, the relative catalytic ratios of 7:Zn^{2+}, 5:Zn^{2+} and 4c:Zn^{2+} is 3:1.7:1.

Without knowledge of the nature of the active species it is difficult to ascertain what features are important for rate enhancement in a given case. X-ray crystallographic analysis of 4c:Zn^{2+} [20] indicated that the three di*iso*propylimidazoles encapsulate the Zn^{2+} ion in such a restrictive way (Eq. 16) that the exchange of reactants which is likely involved during the catalytic cycle, at the remaining metal site(s) would produce a 5-coordinate complex which is severely buttressed.



(16)

R = *iso*propyl

x = di*iso*propylimidazole

Since at least one of the postulated mechanisms for carbonic anhydrase[16] involves an association of both H_2O (OH) and CO_2 in a 5 coordinate complex during the catalytic cycle it appears likely that one important feature

TABLE 4

Effects of ligand:Zn^{2+} Concentration on k_{obs}[a,b] for 5

[Catalyst]	k_{obs}[c]
0.5×10^{-4}	0.78
1.75×10^{-4}	0.96
2.5×10^{-4}	1.08
3×10^{-4}	1.09
5×10^{-4}	1.46

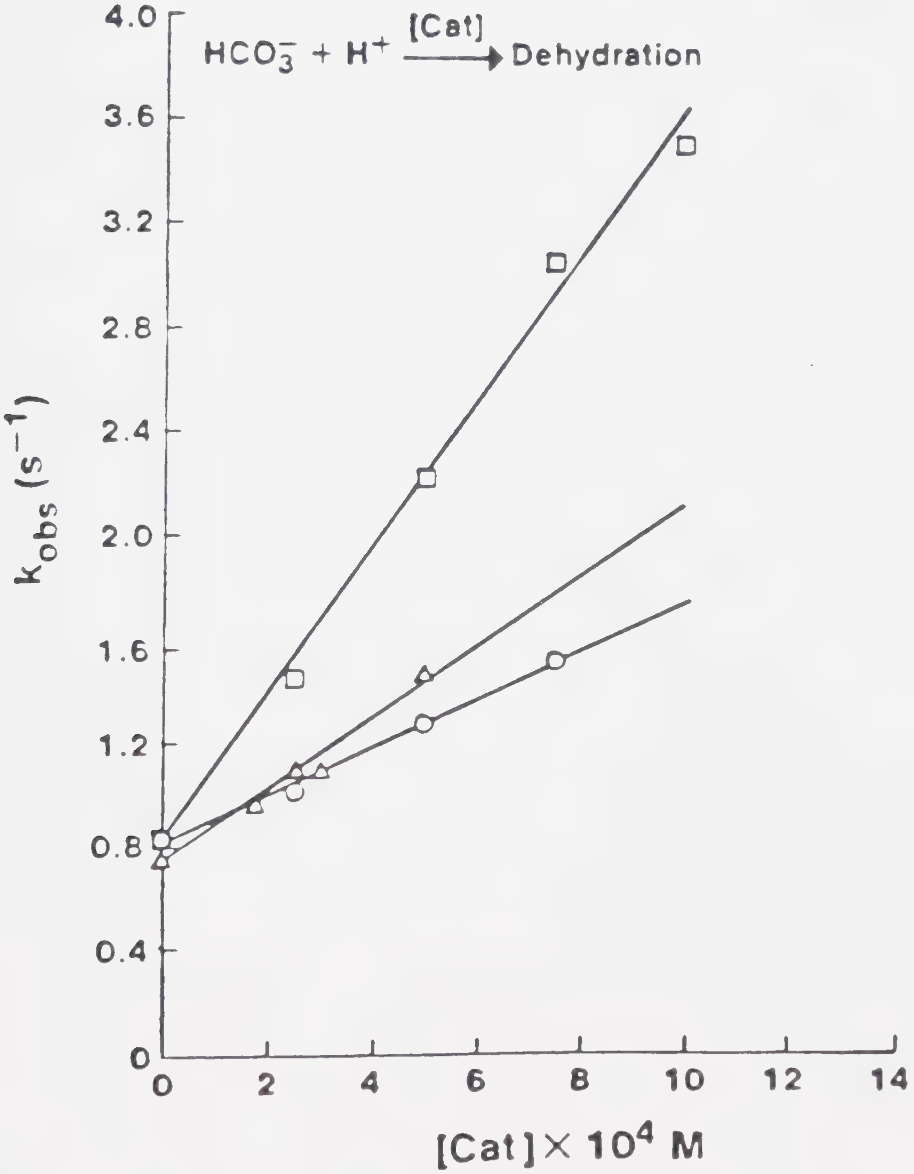
a.) 80% EtOH/H_2O; 25.0 ± 0.2°C; 1×10^{-3}M $NaHCO_3$; 2.5×10^{-2}M HEPES; ionic strength 0.2M ($NaClO_4$).

b.) Followed by monitoring the appearance of bromocresolpurple anion at 580 nm, BCP = 5×10^{-4}M.

c.) Values of k_{obs} ± 0.03 units.

FIGURE 10

k_{obs} for HCO_3^- $\rightleftharpoons$ CO_2 equilibration as a function of [catalyst]. 80% EtOH:H_2O, 0.2M $NaClO_4$; $5x10^{-4}$ in bromocresol purple indicator; $1x10^{-3}$ M $NaHCO_3$, $2.5x10^{-2}$ M HEPES; □ - 7:Zn^{2+}, initial pH 6.45; ○ - 4c:Zn^{2+}, pH 6.45; △ 5:Zn^{2+}, pH 6.55



in any of these model catalysts should be accessibility of the reagents to the metal surface. Assuming that, **4c**:Zn^{2+} has such a restrictive cavity then substituting one of the di*iso*propyl imidazole groups in the compound with a less bulky group to form **5**:Zn^{2+} should produce a cavity with greater accessibility of the reagents and at the same time retain low coordination numbers around the metal. Since **5**:Zn^{2+} is more active than **4c**:Zn^{2+}, the expectation seems to be borne out, however, the increase in k_{obs} is not as large as expected leading to the conclusion that some other factors may be involved in catalysis.

Even though it contains a hydroxyethyl group that was expected to mimic the activity influencing threonine OH group associated with the enzyme's active site[16] **6**:Zn^{2+} proved to be less active than **5**:Zn^{2+}. The NMR spectrum of **6**:Zn^{2+} clearly indicates that the hydroxyethyl group can be close to the Zn^{2+} (as would be required for mimicking the possible interaction of OH in the catalytic process). However, the observation of minimal activity tends to indicate that none of the species present is extremely active and therefore evidence cannot be provided for hydrogen bonding assistance in this model.

The fact that the 7:Zn^{2+} complex is more active yet can be rationalized by considering the greater flexibility of the ligand n-propyl groups as compared to the *iso*propyl ones. This could lead to a greater accessibility to the metal surface. In addition, from a molecular model of the complex it can be seen that many of the possible conformations extend the n-propyl group further into the solution producing a slightly deeper hydrophobic pocket in which the catalytic event can occur. That the activity is larger tends to support idea that hydrophobocity is an important catalytic requirement.

Since monovalent anions are known to inhibit the activity of carbonic anhydrase[1,10], noncompetively for CO_2 hydration and competitively for HCO_3^- dehydration, studies were undertaken to test whether they also diminished the activity of these catalysts. The experiments were performed at the optimal pH value for each compound and the data for 5:Zn^{2+} and 7:Zn^{2+} are given in Table 5.

It can be concluded that these anions are inhibitors as had been found for 4c:Zn^{2+} [45]. However, with increasing $[A^-]$ k_{obs} values for both 5:Zn^{2+} and 7:Zn^{2+} asymptotically approaches limiting values higher than those observed for the uncatalysed reactions. This could indicate that a ternary complex is formed between catalyst and anion and that this complex is still catalytically

TABLE 5

Effects of Added Monovalent Anions on k_{obs}[a].

$k_{obs}(s^{-1})$

[$\underline{5}$:Zn^{2+}] = 5×10^{-4}M, pH = 6.5[b]

$\frac{[\text{Anion}]}{[\text{Catalyst}]}$	NaCl	NaBr	NaI	$NaNO_3$
0.0	1.43	1.43	1.43	1.43
0.5	0.98	--	--	--
1.0	0.79	1.04	1.15	1.19
5.0	0.78	--	--	--

$k_{obs}(s^{-1})$

[$\underline{7}$:Zn^{2+}] = 5×10^{-4}M, pH = 6.45[c]

$\frac{[\text{Anion}]}{[\text{Catalyst}]}$	NaCl	NaBr	NaI	$NaNO_3$
0.0	2.21	2.21	2.21	2.21
0.4	1.6	1.74	1.94	1.89
0.8	1.4	--	--	--
1.0	--	1.63	--	1.48
1.6	1.06	--	1.59	--
2.0	--	1.45	--	--
5.0	1.08	1.33	1.47	1.49

a) Conditions as defined in Table 2

b) Uncatalysed k_{obs} invariant with anion concentration; 0.70 s^{-1}

c) Uncatalysed k_{obs} invariant with anion concentration; 0.80 s^{-1}

viable. No effect of added anion is seen on k_{obs} for the uncatalyzed blank reaction.

Equation 17 represents a simple scheme to accommodate the results from the inhibition experiments. The fact that the values of k_{obs} for 7:Zn^{2+} (Table 6) increase with bicarbonate concentration even in the presence of Cl^- indicates either a competition in which the inhibitory anion can be displaced from the zinc by increasing bicarbonate concentration or that a new active ligand coordinated complex is produced ($A^- -Zn^{2+}-HCO_3^-$).

However, the scheme becomes complicated due to the fact that bicarbonate has been shown to be an inhibitor for the enzymatic CO_2 hydration[24] and could behave in a similar fashion with these ligands.

$$\begin{tikzcd}
A^- + L{:}Zn^{2+} + HCO_3^- \arrow[r, shift left, "k_1"] \arrow[d, shift left, "k_3"] & A^- + L{:}Zn^{2+}-HCO_3^- \arrow[l, shift left, "k_{-1}"] \arrow[r, shift left, "k_2 {[H^+]}"] \arrow[d, shift right, "k_7"'] & A^- + L{:}Zn + CO_2 + H_2O \arrow[l, shift left, "k_{-2}"] \arrow[d, shift left, "k_{-6}"] \\
L{:}Zn^{2+}{:}A^- + HCO_3^- \arrow[u, shift left, "k_{-3}"] \arrow[r, shift left, "k_4"] & L{:}Zn^{2+}\begin{array}{l} \diagup A^- \\ \diagdown HCO_3^- \end{array} \arrow[l, shift left, "k_{-4}"] \arrow[u, shift right, "k_{-7}"'] \arrow[r, shift left, "k_5 {[H^+]}"] & L{:}Zn^{2+}A^- + CO_2 + H_2O \arrow[l, shift left, "k_{-5}"] \arrow[u, shift left, "k_6"]
\end{tikzcd} \qquad (17)$$

A study was undertaken to determine whether changes in $NaHCO_3$ affected k_{obs} in a way which might be indicative of a saturation phenomenon. Table 6 provides the data obtained for 5:Zn^{2+}, 7:Zn^{2+} and 4c:Zn^{2+} at their optimal rate pH values.

TABLE 6

Effect of $[NaHCO_3]$ on k_{obs}[a]

$[NaHCO_3]\times10^4$	$\underline{4c}:Zn^{2+}$ [b] $5 \times 10^4 M$		$\underline{5}:Zn^{2+}$ [b] $5 \times 10^4 M$		$\underline{7}:Zn^{2+}$ [c] $2.5 \times 10^4 M$	
	no Cl^-	$5\times10^4 M$ Cl^-	no Cl^-	$5\times10^4 M$ Cl^-	no Cl^-	$5\times10^4 M$ Cl^-
3	0.95	0.63	1.0	--	1.29	0.9
5	--	0.65	1.07	--	1.53	1.03
7.5	1.05	--	1.27	--	1.55	--
10	1.10	0.69	1.42	0.72	1.58	1.15
17.5	1.16	--	1.59	--	1.71	1.25

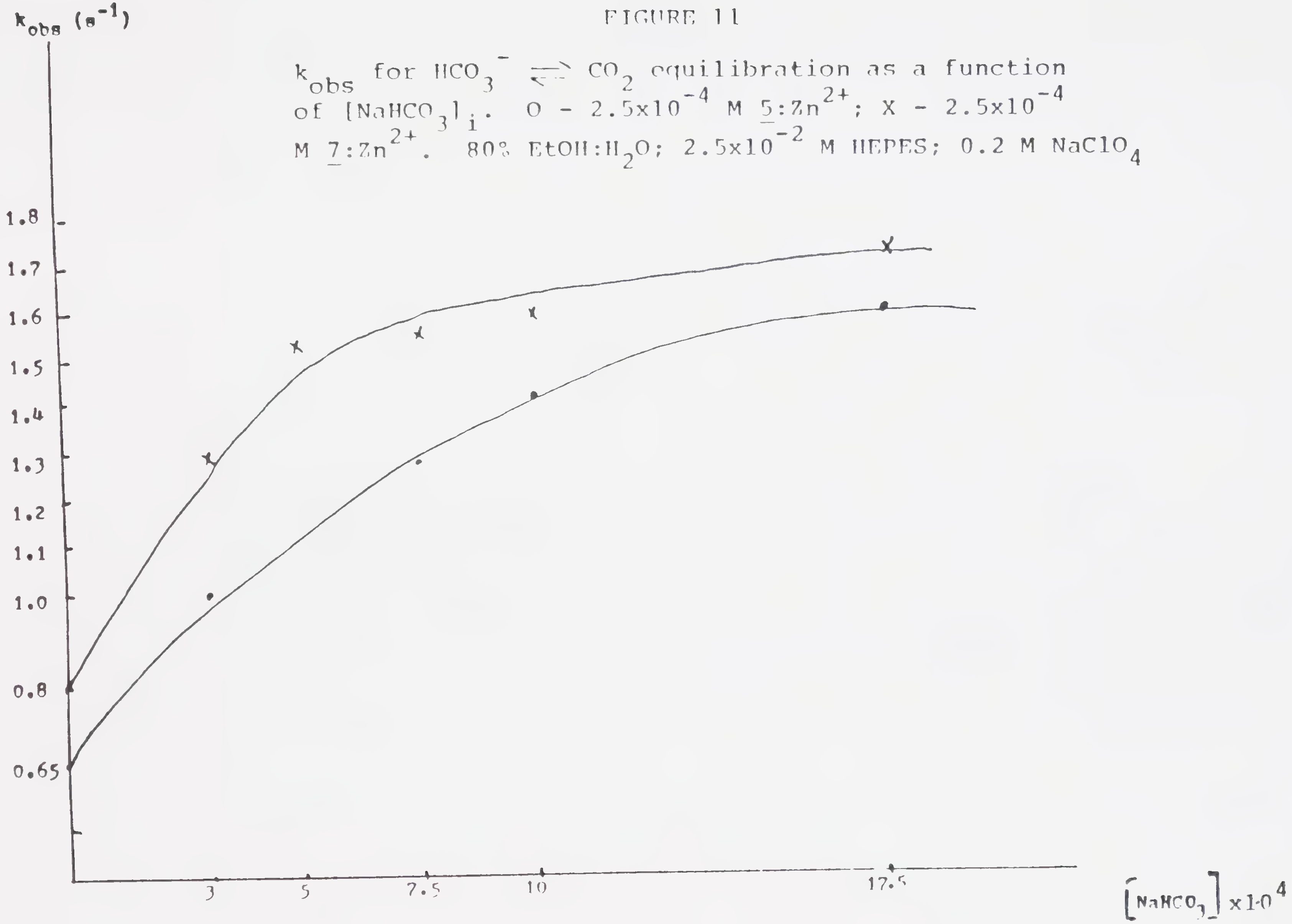
a) Conditions as defined in Table 2

b) pH = 6.55; k_{obs} in the absence of catalyst = 0.65 s^{-1} and is invariant with $[NaHCO_3]$ and added Cl^-.

c) pH = 6.45; k_{obs} in the absence of catalyst = 0.80 s^{-1} and is invariant with $[NaHCO_3]$ and added Cl^-.

FIGURE 11

k_{obs} for $HCO_3^- \rightleftharpoons CO_2$ equilibration as a function of $[NaHCO_3]_i$. O - 2.5×10^{-4} M 5:Zn^{2+}; X - 2.5×10^{-4} M 7:Zn^{2+}. 80% EtOH:H_2O; 2.5×10^{-2} M HEPES; 0.2 M $NaClO_4$



The range of concentration of HCO_3^- over which these experiments were performed is quite limited due to the fact that lower concentrations of bicarbonate ion produced a very small change in absorbance, while higher concentrations than the ones used led to pH changes of 0.1 units or more during the kinetic runs making the values of k_{obs} meaningless. The fact that in the absence of ligand k_{obs} does not depend on bicarbonate concentration tend to verify that some interaction is occuring between bicarbonate and ligand and that this accelerates the attainment of equilibrium for the reaction. By plotting k_{obs} vs. $[HCO_3^-]$(Figure 11), a non linear plot is obtained which could be taken to be indicative of some sort of saturation phenomenon.

The traditional approach to enzyme catalysis involves the Michaelis-Menten model that takes into account the idea that an enzyme interacts with a substrate reversibly to form the noncovalent ES complex which can then either undergo chemical reaction to products or dissociate back to starting materials[53]. The steady state assumption formulated by Briggs and Haldane[54] can then be introduced to simplify the system. However, this generally requires that enzyme be present in catalytic amount ([S]>> total

enzyme)[53]. Unfortunately the scheme depicted for these catalysts cannot be fit to the Michaelis-Menten model and any attempt to simplify the equations by the Briggs-Haldane assumption is invalidated since the substrate concentration was similar to the catalyst concentration.

Up to this point all kinetic studies refer to the value of k_{obs} which as previously stated is a summation of forward and reverse rate constants. The mathematical treatment could be simplified if the reversibility of the reaction is suppressed by studying only the first few percent when the concentration of product tends to zero.

Initial rate experiments were performed in an attempt to obtain independent values of k_f and k_r. These were conducted from both the dehydration and hydration directions by evaluating the slopes of absorbance vs. time plots for the first 5% of the reaction (after eliminating the first part of the curve corresponding to the mixing of the reactants.)

These experiments were performed following the procedures of DeVoe and Kistiakowski and Gibbons and Edsall[50]. For calculation of the initial rate of the reaction it is necessary to know the effect of the addition of H^+ ions on the absorbance of the buffer-indicator system. One way to determine the nearly linear relationship

between these two quantities is to titrate an aliquot of buffered indicator solution with acid or base and determine the absorbance change. The slope of the plot of $[H^+]$ vs. absorbance is defined as the "buffer factor". The buffer factor may also be calculated for a given system assuming that the indicator does not contribute to the buffering capacity of the solution[26].

For dehydration of bicarbonate the rate of disappearance of bicarbonate ion or appearance of indicator anion can be expressed as in equation 18a where $d[H^+] / d(Abs_{580})$ is the aforementioned experimental buffer factor.

$$(18a) \quad \frac{-d[HCO_3^-]}{dt} = \frac{-d[H^+]}{dt} = \frac{d[A]}{dt} = \frac{d(Abs_{580})}{dt} \cdot \frac{d[H^+]}{d(Abs_{580})} = k_f[HCO_3^-]_i$$

$$(18b) \quad k_f = \frac{d(Abs_{580})}{dt} \cdot \frac{d[H^+]}{d(Abs_{580})} / [HCO_3^-]_i$$

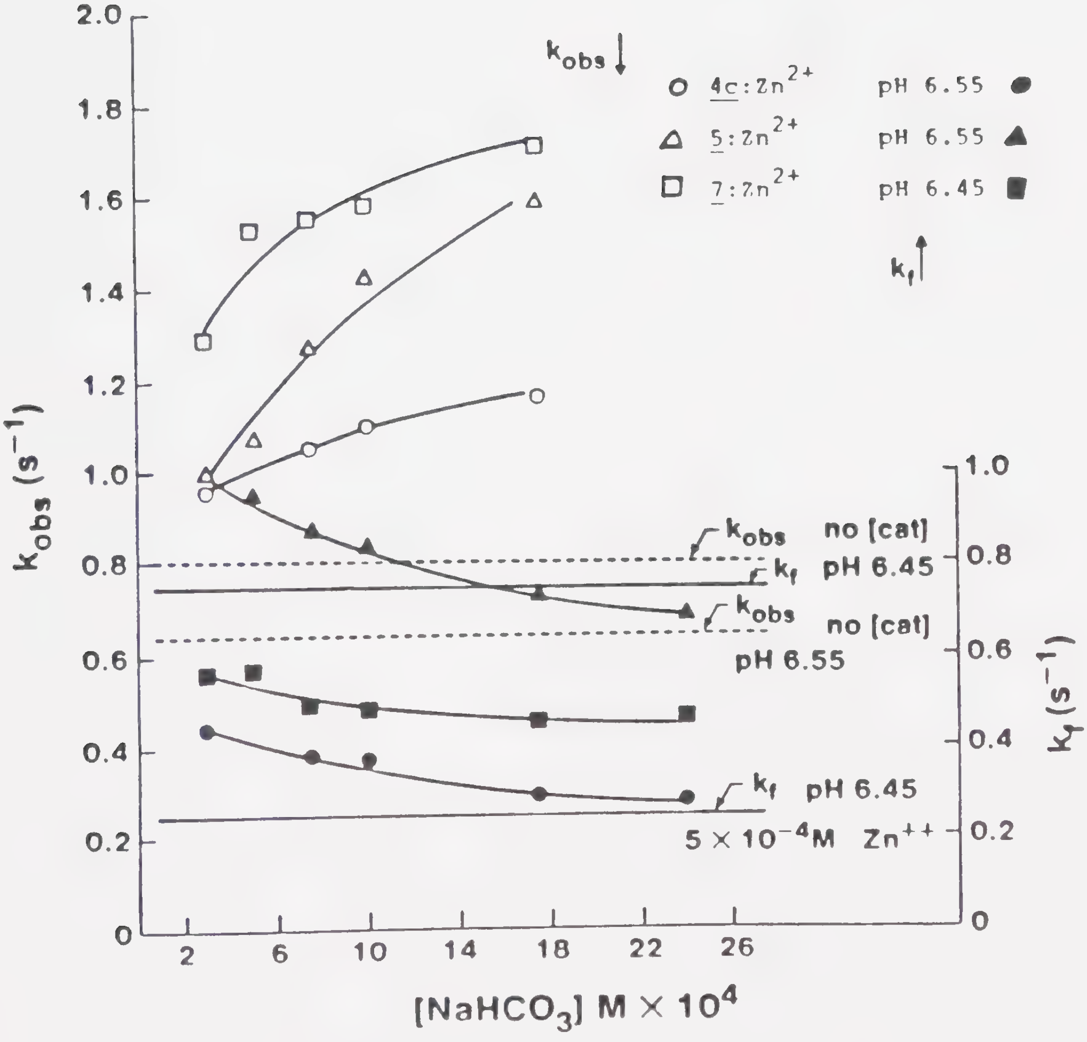
Table 7 shows the results of the determinations for bicarbonate dehydration with $\underline{5}$:Zn^{2+}, (the other ligands showed similar trends). Figure 12 displays the results while Table 8 shows the results obtained for CO_2 hydration for $\underline{5}$:Zn^{2+}.

In the absence of added ligand or Zn^{2+}, it is observed that the apparent k_f is invariant with increasing $[HCO_3^-]_i$. However, the results obtained in the presence of Zn^{2+} show that k_f is reduced even though k_{obs} remains

FIGURE 12

k_{obs} (open symbols) and apparent k_f (closed symbols) as a function of initial $[NaHCO_3]$. Dashed horizontal lines represent k_{obs} determined with no added catalyst at pH 6.45 and 6.55 while the two continuous horizontal lines represent apparant k_f in the presence of 5×10^{-4} M Zn^{2+} alone, pH 6.45. 80% EtOH:H_2O; 0.2 M $NaClO_4$; 5×10^{-4} M bromocresol purple; 2.5×10^{-2} M HEPES

FIGURE 12


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
kobs (s-1)
kobs ↓
○ 4c:Zn2+ pH 6.55 ●
△ 5:Zn2+ pH 6.55 ▲
□ 7:Zn2+ pH 6.45 ■
kf ↑
kobs no [cat]
kf pH 6.45
kobs no [cat]
pH 6.55
kf pH 6.45
5 × 10-4M Zn++
1.0
0.8
0.6
0.4
0.2
0
kf (s-1)
2
6
10
14
18
22
26
[NaHCO3] M × 104

unchanged from that seen in the absence of Zn^{2+}. This observation requires for k_r to increase in order to maintain a constant value of k_{obs} and this leads to the unlikely conclusion that the $HCO_3^- \rightleftharpoons CO_2$ equilibrium constant is changed in the presence of Zn^{2+} but in some way independent of the $Zn^{2+}/[HCO_3^-]$ ratio. In the presence of ligand:Zn^{2+} the observation appears to be that k_f actually diminishes with increasing $[NaHCO_3]_i$ even though k_{obs} is increasing. These results lead to the unlikely conclusion that K_{eq} in the presence of catalyst for $HCO_3^- \rightleftharpoons CO_2$ varies as a function of $[HCO_3^-]$ and that in the high concentration limit lies more to the side of bicarbonate even if the bulk of HCO_3^- in solution is not associated with the complex and should proceed to CO_2 at the same rate as when the catalyst is not present. Furthermore, the values of k_f obtained for CO_2 hydration show only a slight increase as a function of $[CO_2]$ that does not account for the trends in k_{obs}. This could be due to an experimental artifact and thus a closer study of the method employed was undertaken. The following information was thus obtained:

1) Some authors[26] have noted that the buffer factor changes to some extent during the course of the reaction but this can be minimized by using buffers and indicators that have nearly identical pH values.

TABLE 7

Initial Rates for Ligand 5:Zn^{2+} [a]

$[NaHCO_3] \times 10^4 M$	k_{obs} [b] (s^{-1})	Initial Rate $\times 10^4 (M.s^{-1})$	k_f [c,d,e,f] (s^{-1})
3	1.0	3.12	1.0
5	1.07	4.7	0.95
7.5	1.27	6.5	0.87
10.0	1.27	8.4	0.84
17.5	1.50	12.8	0.73
25.0	--	16.9	0.68

a) Temp = 25.0 ± 0.2°C; [BCP] = 5×10^{-4}M; Ionic strength = 0.2M ($NaClO_4$); 80% EtOH/H_2O; pH=6.55; HEPES=0.025M; [Catalyst]=5×10^{-4}M.

b) k_{obs} values ± 0.03.

c) k_f values for uncatalyzed reaction=0.57 s^{-1}.

d) k_f values in the presence of 5×10^{-4} chloride anion = 0.65 s^{-}

e) k_f calculated as in eq. 18b.

f) k_f values in the presence of Zn^{2+} (no ligand) = 0.25 s^{-1}.

TABLE 8

Initial Rates For CO_2 Hydration for Ligand 5[a,b]

$[CO_2]\times 10^3$	Initial Rate $\times 10^4$ ($M.s^{-1}$)	k_f (s^{-1})
2	0.66	0.06
5	4.9	0.19
10	11.3	0.22

a) Temp = 25.0 ± 0.2°C; [BCP] = 5×10^{-4}M; Ionic strength = 0.2M ($NaClO_4$); 80% EtOH/H_2O; pH=6.55; HEPES=0.025; Catalyst=5×10^{-4}M.

b) k_{obs} value for catalyzed reaction when $[CO_2]=2 \times 10^{-3}$M, 1.41 s^{-1}.

2) In aqueous solutions buffer factors can be calculated if the exact pK's of the buffer and indicator are known as well as the change in molar extinction coefficient between acidic and basic forms of the indicator. However, since the solutions required here for catalyst solubility are highly organic in nature, the buffer factor must be determined experimentally.

Visible absorption spectra of buffered solutions containing BCP, $Zn(ClO_4)_2$ and $NaHCO_3$ were monitored as a function of small aliquots of $HClO_4$. Figure 13 shows that once equilibrium is attained the maximum absorbance changes occur at 600 nm reflecting (as expected) the change in BCP anion concentration. Control experiments indicate that the various buffer and indicator equilibria are established rapidly. However, in the presence of $NaHCO_3$ spectral changes depend upon time since the dehydration process takes about 10 sec. to attain equilibrium.

A stopped flow experiment was performed in which absorbance vs. time points were taken at various wavelengths. Figure 14 shows that when two solutions containing indicator and metal and HCO_3^- are rapidly combined the maximal absorbance changes are at 570-580 nm (which is the wavelength used to monitor the reaction) thus indicating that a transient intermediate is formed and that

FIGURE 13

Visible absorption spectra showing the equilibrium situation for buffered solutions of BCP as a function of added 1 μl aliquots of o.1 M $HClO_3$. (2.5×10^{-2} M HEPES; 2.5×10^{-4} M BCP; 2.5×10^{-4} M $Zn(ClO_4)_2$; $[NaHCO_3]$ initially 2.5×10^{-3} M; 0.2 M $NaClO_4$; 80% EtOH:H_2O. a.u.=arbitrary units.

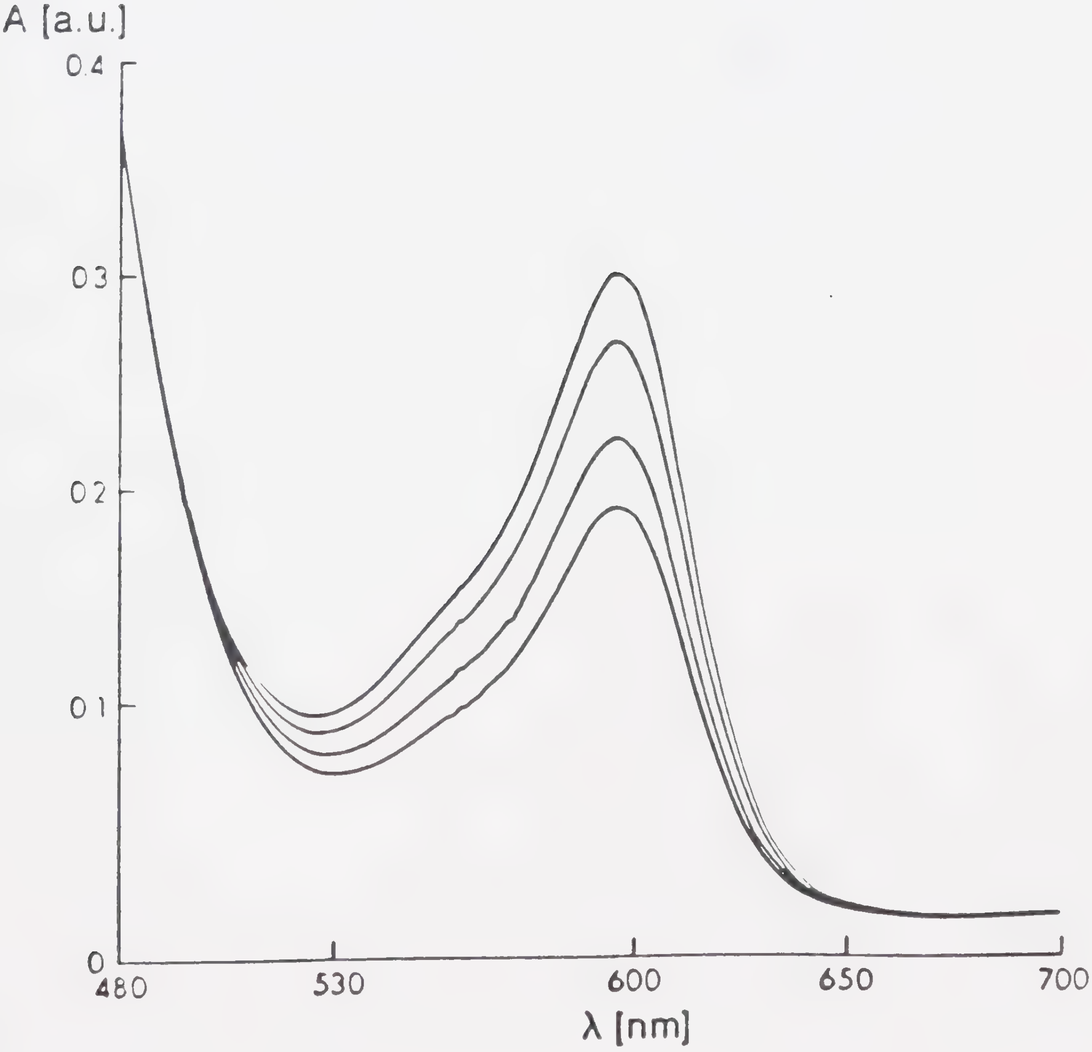
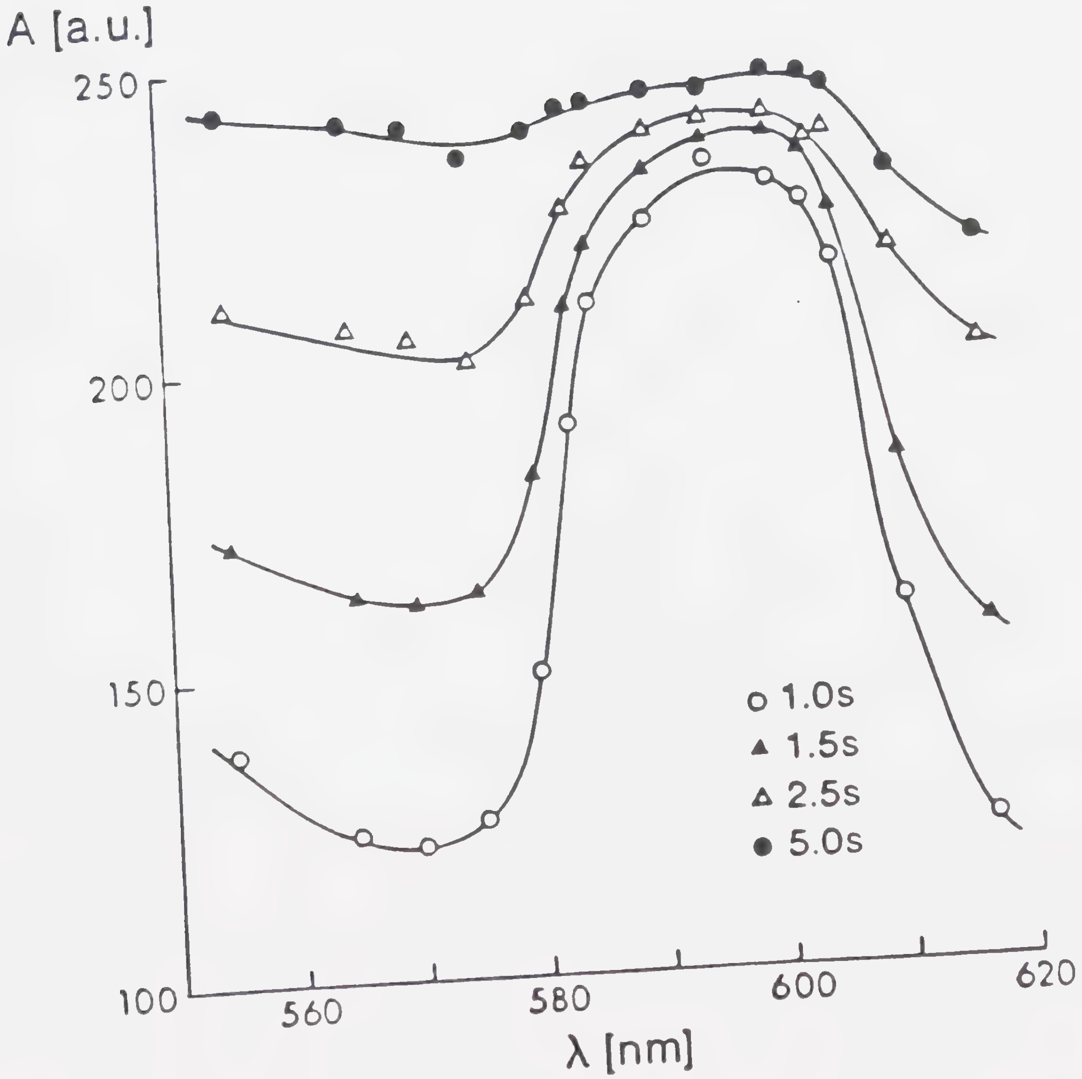


FIGURE 14

Transient absorption spectra of a buffered solution of $Zn(ClO_4)_2$ and HCO_3^- after rapid mixing with a buffered solution of BCP (same initial conditions as in Figure 13 as a function of time).



it has different spectral characteristics than the BCP and Zn^{2+} complex. These observations preclude the exact evaluation of k_f under the conditions employed for the kinetic studies since the buffer factor of the indicator is therefore dependent upon the presence of HCO_3^-. It must be stressed however, that the values obtained for k_{obs} are still valid since this is a measure of the rapidity with which the entire system attains equilibrium and its value is independent of wavelength.

Based on the experiments performed and the limitations mentioned above only a qualitative discussion of the system can be presented. A very general scheme has been devised to account for the different results. (Scheme 3). Since there is evidence that indicates that at pH's and anionic conditions where the catalysts show their maximal activities in promoting $HCO_3^- \rightleftharpoons CO_2$ equilibration, the complexes are undergoing rapid ligand exchange, several forms of the complex must be present in solution in a dynamic equilibrium. In order to keep the scheme as simple as possible, only three of these forms are presented in each column. This should not be interpreted as a dismissal of other forms which may be catalytically active. The major distinction in progressing down a given column is in the state of coordination number of Zn^{2+} and number of associated solvent

molecules as well as the state of protonation of the ligand.

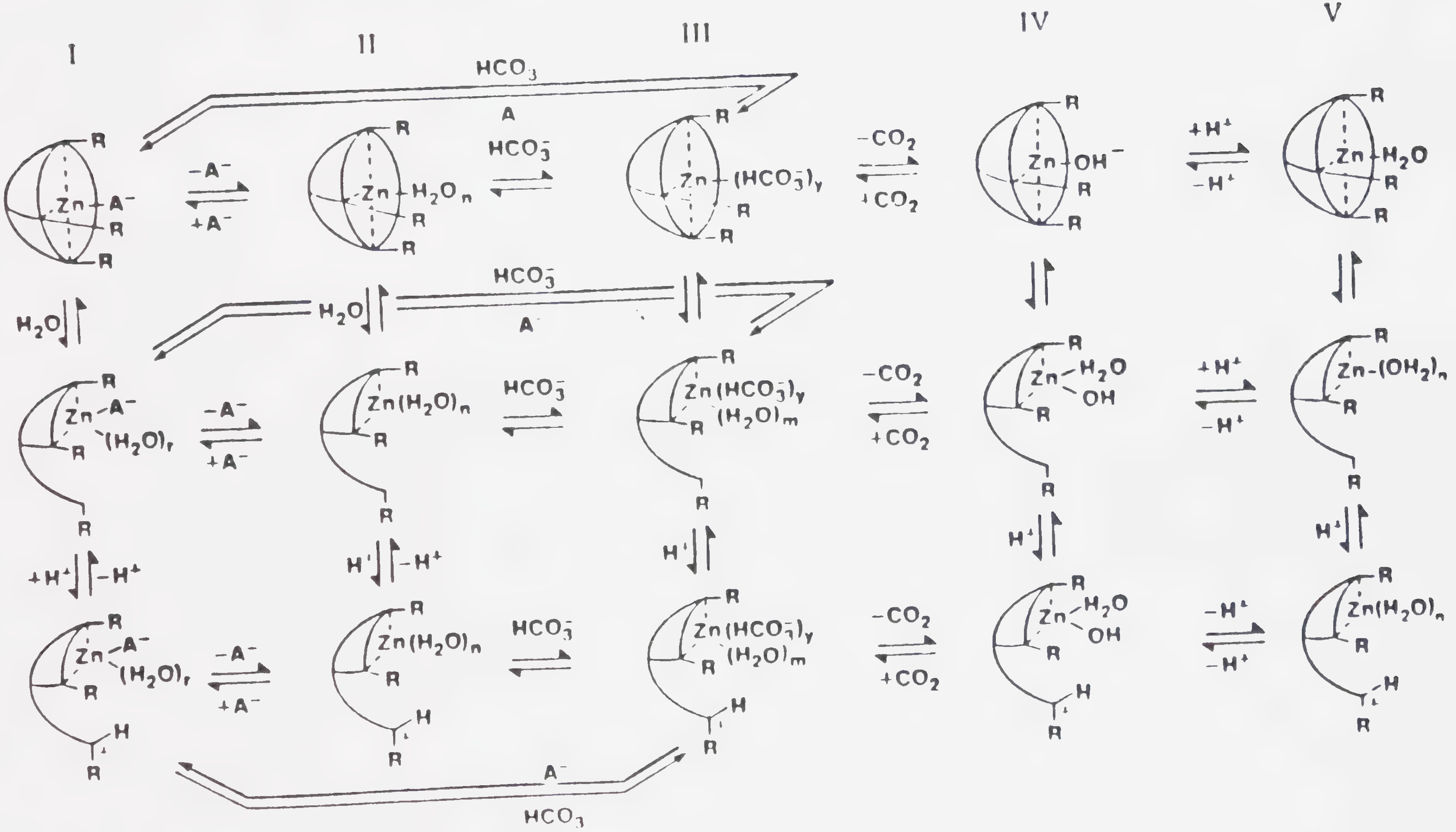
Disassociation of an imidazole not only increases accessibility of species from the solution to the Zn^{2+} but could provide a closely situated base to assist in proton transfer (a role that some groups have assigned to His 64)[12]. When present in solution, HCO_3^- can associate with any (or all) of the coordinated $L:Zn^{2+}$ species to form a set of ternary complexes (column III) from which expulsion of CO_2 occurs. Formation of $L:Zn^{2+}-OH^-$ species is suggested after CO_2 elimination and this undergoes reprotonation to yield a metal bound H_2O.

Inhibitory anions tend to perturb the equilibria towards the $L:Zn^{2+}-A^-$ ternary complexes. These not only have perturbed acid-base equilibria of the ligand-$Zn^{2+}-H_2O$, but also resist displacement by HCO_3^- both factors leading to reduced catalytic activity.

II - F CONCULSIONS

1) The three phosphines studied bind in a 1:1 relationship to zinc. The complexes of two of them (5 & 6) experience a fast dynamic site exchange of the diisopropyl imidazoles on the NMR timescale while that of the third (7) seems to be a rigid tridentate complex. The complex formed by ligands 6 and 7 is highly dependent on the counterion.

Scheme 3 (Metal charges omitted for simplicity)


I
II
III
IV
V
HCO₃
A
R
Zn
A⁻
−A⁻
+A⁻
H₂Oₙ
HCO₃⁻
(HCO₃⁻)ᵧ
−CO₂
+CO₂
OH⁻
+H⁺
−H⁺
H₂O
H₂O
A⁻
Zn(H₂O)ₙ
Zn(HCO₃⁻)ᵧ
(H₂O)ₘ
(H₂O)ᵣ
OH
Zn-(OH₂)ₙ
H⁺
H
HCO₃

2) The complexes catalyse the equilibration of HCO_3^- $\rightleftharpoons$ CO_2 in the order $\underline{7}:Zn^{2+} > \underline{5}:Zn^{2+} > \underline{4c}:Zn^{2+} > \underline{6}:Zn^{2+}$.

3) These results are consistent with the idea that steric factors are important in the design of models for carbonic anhydrase since ligands 5 and 7 accelerate the reaction more than 4c which offers a somewhat more restrictive cavity.

4) The presence of a hydroxyethyl group in compound 6 does not accelerate the reaction as would be expected if this group approximates the threonine OH which is said to be important in the enzyme catalyzed process. This observation could be due to the fact that the hydroxyethyl group could be on the distal side of a bound imidazole or that the hydroxyethyl imidazole itself was not bound. ^1H NMR experiments on the mode of Zn^{2+} binding by 6 showed that the imidazole 4(5) H shifts to lower field in the presence of Zn^{2+} as expected if the imidazole is associated but broadens considerably indicating some dynamic process. However, since the activity exhibited by $\underline{6}:Zn^{2+}$ is much lower than for $\underline{5}:Zn^{2+}$ it cannot be contended that any of the configurations of $\underline{6}:Zn^{2+}$ is tremendously active.

5) The system used to monitor the reaction cannot be used to determine initial rates since there appears to be a short lived intermediate complex between bicarbonate and the indicator used.

6) The next step in constructing a carbonic anhydrase model should be to increase the hydrophobicity of the cavity by having different groups substituted to the imidazole such as phenyl and other substituted aromatic groups.

CHAPTER III

EXPERIMENTAL

III - A SYNTHESES

Routine IR and ^{1}H NMR spectra were obtained with a Nicolet FTIR spectrophotometer and a Bruker WP-80 spectrometer respectively. Mass spectra were obtained on a MS-50 spectrometer.

All the ethereal solvents used were distilled from Na and benzophenone, syringe techniques were employed throughout. For n-Bu Li reactions, this was titrated prior to use (absolute ethanol, phenantrolene indicator) and flasks were oven dried and nitrogen flushed.

Ethyl Isobutyrate

This was prepared by the procedure outlined by Vogel[55], b.p. 110-111°C (lit.[55] b.p. 111°C), ^{1}H NMR ($CDCl_3$): δ1.25 (9H,m), δ2.5 (1H,m), δ4.2 (2H,q). Yield 66%.

Isobutyroin

The procedure of Snell and McElvain[56] was employed. B.p. 96°C @ 27 mm; (lit.[56] b.p. 80-86°C @ 12 mm); ^{1}H NMR ($CDCl_3$) δ0.7 (6H,d), δ1.1 (6H,d), δ2.2 (1H,m), δ2.75 (1H,m), δ3.4 (1H,d), δ4.1 (1H,q). Yield 75.4% (lit.[56] 70%).

4,5-Di-*iso*-propylimidazole

This was prepared by the procedure of Bredereck and Thelig[57]. M.p, 212°C (lit.[57] m.p. 213-214°C); ^{1}H NMR ($CDCl_3$) δ1.3 (12H,d), δ3.1 (2H,m), δ7.5 (1H,s). Yield 97%.

N-(dimethoxymethyl)-4,5-di-*iso*-propylimidazole

A procedure similar to the one reported by Brown and Curtis[45] was followed. 20 g. (0.134 moles) of 4,5-di-*iso*-propylimidazole were mixed with 250 ml of toluene, 56.8 g. (0.54 mol) of trimethyl orthoformate and 2 ml formic acid. The mixture was heated until no more methanol was distillable from the reaction mixture. Excess solvent and orthoformate were removed by rotary evaporation and the resulting red solution was filtered into a flask containing 1 g Na_2CO_3. The mixture was vacuum distilled giving 14.6 g (72.7% overall yield) (lit.[45] yield 80%) of the desired product, b.p. 91°C @ 0.3 mm (lit.[45] b.p. 96-100°C @ 0.5 mm); ^{1}H NMR ($CDCl_3$) δ1.1 (12H,d), δ3.0 (2H,m), δ3.25 (6H,s), δ5.75 (1H,s), δ7.58 (1H,s).

N-(diethoxymethyl)imidazole

The procedure of Brown and Curtis[45] was followed, b.p. 82°C @ 0.3 mm (lit.[45] b.p. 52°C @ 0.02 mm), ^{1}H NMR ($CDCl_3$) δ1.2 (6H,t), δ3.6 (4H,q), δ6.1 (1H,s), δ7.3 (2H,d), δ7.6 (1H,s). (Yield 75%, lit.[45] 80%).

Bis-(4,5-di-*iso*-propyl-2-imidazolyl)-2-imidazolylphosphine(5)

To a stirred solution of 16.88 g. (0.074 mol). freshly distilled N-(dimethoxymethyl)-4,5-di-*iso*-propylimidazole in 150 ml dry ether held at -40°C was added via syringe 45.32 ml (0.0747 mol.) of 1.65 N *n*-Bu Li in hexane at such a rate that the temperature did not exceed -40°C. After the addition the resulting yellow solution was stirred an additional 20 minutes and then cooled to -60°C. This solution was then transferred by syringe to a second flask containing a stirring solution of 5.13 g (0.037 mol.) freshly distilled PCl_3 in 150 ml dry THF also held at -60°C. The rate of addition was adjusted so that the temperature in the second flask remained at less than -55°C. After addition the mixture was allowed to come to room temperature overnight with stirring. In the morning in a third flask the anion of N-(diethoxymethyl)imidazole was prepared by addition of 31.7 ml (0.052 mol.) of 1.65 N *n*-Bu Li to 8.9 g. of the imidazole (0.052 mol.) in 150 ml dry THF held at -40°C. After the mixture was stirred for 20 minutes to ensure complete deprotonation, it was transferred by syringe to the flask containing chloro-bis-(N-(diethoxymethyl)-4,5-di-*iso*-propyl-2-imidazolyl)phosphine held at -40°C. The resultant coral-colored mixture was allowed to come to room temperature and stirred over the weekend.

Workup of the product was carried out by extracting the reaction mixture with concentrated NH_4OH, separating the organic layer, and removing the solvent by rotary evaporation. The NH_4OH layer was extracted with 3x100 ml $CHCl_3$ and the combined chloroform extracts were stripped of solvent, the residue being combined with that from the ether layer. Final deblocking was accomplished by refluxing the combined residues with 150 ml of 1:1 methanol: water for 45 minutes. After cooling the mixture, 7.0 g. of yellow powder was collected by filtration and washed with ether to yield 4 g of the crude product. Recrystallization was effected by dissolving the crude product in methanol and adding water until the solution became turbid. After refrigeration for 24 hours, 3.6 g of pure product (overall yield 24%) were obtained, m.p. 194-195°C; ^1H NMR (CD_3OD) δ1.2 (24H,d of d), δ3.1 (4H,m), δ7.1 (2H,s), Anal. calcd. for $C_{21}H_{31}N_6P$:C,63.00;H,8.25;N,21.00; Found:C,62.97; H,8.39;N,20.98. Mass spectrum; m/e calculated for $C_{21}H_{31}N_6P$: 400.2504, found 400.2504; IR (CCl_4 cast): 2899.11, 2801.78, 2596.9, 1237.8 cm^{-1}.

Bis-(4,5-di-*iso*-propyl-2-imidazolyl)-4(5)-hydroxyethyl-2-imidazolyl phosphine (6)

8.7 g of N-dimethoxymethyl-4,5-di-*iso*-propylimidazole (0.038 mol.) in dry THF was converted to the 2-lithio

derivative by treatment with 24.7 ml of 1.56 N n-Bu Li. The anion was then added via syringe at -60°C to 2.72 g (0.019 mol.) of freshly distilled PCl_3 in 100 ml dry THF at such a rate that the temperature did not exceed -60°C. The mixture was allowed to come to room temperature overnight with stirring. In the morning, 19.0 ml (0.029 mol.) of 1.56 N n-Bu Li was added to 5.0 g (0.029 mol.) of the cyclic amide acetal of 4(5)-hydroxyethylimidazole[58] (structure #8 in Scheme 1) in 100 ml dry THF cooled to -40°C. The resultant red solution was stirred at -40°C for 20 minutes and then transferred by syringe to the contents of the first flask which were cooled to -40°C. The mixture was allowed to come to room temperature overnight giving an orange-red solution that was stirred an additional 24 hours. Workup consisted of evaporating the solvent and adding both $CHCl_3$ and 100 ml of concentrated NH_4OH. The NH_4OH layer was extracted with chloroform and the combined organic layers were washed with saturated NaCl. After rotary evaporation of the volatiles, the crude residue was refluxed with 100 ml of 1:1 methanol:water to effect deprotection. Evaporation of the methanol yielded a brown paste that was dissolved in dichloromethane, the insoluble material being filtered off. Evaporation of the solvent left 7.2 g of a brown solid which was triturated with ether to yield 3 g of

a yellow solid. Recrystallization from methanol:water as for 5, gave 2.0 g of pure product (overall yield 22.7%) m.p. 187-188°C,; ^{1}H NMR ($CDCl_3$) δ1.25 (24H,d), δ2.75 - 3.25 (6H,m), δ3.78 (2H,t), δ6.91 (1H,s); Anal. calcd. for $C_{23}H_{37}N_6OP$:C,62.16;H,8.39;N,18.91. Found:C,62.09;H,8.39; N,18.82. IR ($CHCl_3$ cast): 3126.6 (br), 2961.29, 2869, 1461, 1389, 1059 cm^{-1}; Mass spectrum, m/e calcd. for $C_{23}H_{37}N_6OP$: 444.2771, found 444.27.

III - B POTENTIOMETRIC TITRATIONS

1.) pKa Determinations

These were performed in a jacketed cell kept at 25 ± 0.1°C. Air was excluded from the cell by bubbling a stream of nitrogen (purified by bubbling through a solution of $Ba(OH)_2$ and an 80% Ethanol and 20% water solution) through the solution. The pH was measured using a Radiometer TT2 titrator and PHA 943 B titration module in conjunction with a Radiometer GK2402B combination electrode. The pH was recorded as a function of added 0.1 N NaOH (prepared by dissolving NaOH in water and titrating the resulting solution with standard HCl) delivered by a radiometer ABU 12 autoburette, on a Radiometer SBR 3 titrigraph. Commercially available standard pH4 and pH7 buffers were used to calibrate the pH meter at the beginning of each series of experiments. The ionic strength was held constant by using $NaClO_4$.

However, under these conditions the solvent composition and ionic strength are changing as the titration is being carried out. In a second set of experiments this was avoided by using 0.2 N NaOH, prepared by dissolving a commercially available DILUT-IT analytical concentrate in the adequate volume of an 80% EtOH - 20% water (v/v) solution. The pH meter was calibrated by preparing 10^{-1} and 10^{-4} M $HClO_4$ solutions (titrated with standard NaOH) in 80% EtOH - 20% H_2O with enough $NaClO_4$ to make them 0.2 M. in ionic strength and assigning values of pH 1 and pH 4 to these solutions. For all of the above solutions 95% Ethanol was purified by simple distillation at ambient pressure and was assumed to be the right concentration.

Water used for these experiments was triply distilled from permanganate.

Data were analyzed with a computer version of Simm's method[47]. Reported pKa's are the average of at least three determinations. Typically, for the second set of experiments, the cell would contain 1 ml of 0.025 M ligand in an 80% EtOH - 20% H_2O (v/v) solution 0.2 M in sodium perchlorate, 3.2 ml of a stock solution of 80% EtOH - 20% H_2O (v/v) 0.2 M in sodium perchlorate, and 0.8 ml of a 0.117 M solution of $HClO_4$ in 80% EtOH - 20% H_2O (v/v) with enough $NaClO_4$ to bring the ionic strength to 0.2 M.

2.) Metal Binding Constants

Stock solutions of Co(II) and Zn(II) were prepared from their reagent grade perchlorate salts and were standardized by Murexide[59] or EDTA[60] titrations. Typically, a two fold excess of ligand over metal was titrated as above and the data analyzed with the aid of a computer program supplied by Prof. R. Breslow of Columbia University and modified to be used on a Commodore PET 4032 micro computer. The ordering for metal stability constants ($pK_M{}^{2+}$) for the ligands under study is Zn(II) > Co(II). No second binding constants are observed for these compounds (as expected if the substituents encapsulate the metal sufficiently to inhibit 2:1 complexation). The values reported in Table 1 are the average of three determinations for both sets of conditions.

3.) Complex Titration

80% ethanol:water solutions containing equimolar amounts of ligand and metal and a known amount of nitric acid were titrated with NaOH (0.1M).

III - C NUCLEAR MAGNETIC RESONANCE STUDIES OF Zn(II) COMPLEXES.

Typically 4-5 mg of ligand were dissolved in 0.5 ml of methanol-$\underline{d}_4$ and microliter amounts of either 0.25 M $Zn(ClO_4)_2$ or $ZnCl_2$ in D_2O were added, a spectrum

being recorded after each addition. The spectra were recorded on a Bruker-WH-200 Fourier Transform NMR spectrometer.

III - D Co(II) UV-VISIBLE SPECTRA

These were recorded with a Carey Model 210 spectrophotometer using 1 cm cells. In a typical experiment, a solution of 80% ethanol:water containing equimolar amounts of ligand and $CoCl_2$ was placed in one cell and the spectrum recorded against a reference cell containing 80% ethanol:water. The ionic strength was kept at 0.2 M by addition of NaCl. Spectra were recorded as a function of pH which was changed by adding microliter aliquots of NaOH to the cell.

III - E CATALYTIC STUDIES OF CO_2 HYDRATION AND HCO_3^- DEHYDRATION

Solutions consisting of 80% ethanol:water were used for kinetic runs to ensure solubility. Determinations were performed on a Durrum-Gibson stopped-flow instrument thermostated at 25 ± 0.2°C. Absorbance vs. time traces were digitally stored with a TDI model 1024-C Transient Recorder having 8-bit resolution. Data manipulation and calculations were performed on a Commodore PET Model 4032 microcomputer interfaced to the system. The k_{obs} values reported are those calculated by fitting the

absorbance/time data to a standard first order exponential model using a non-linear least squares technique[61]. The values obtained are the averages of 6 to 10 determinations taken at each given set of conditions.

CO_2 solutions were prepared by bubbling CO_2 through an 80% ethanol:water solution for one hour[21]. The concentration of CO_2 was determined[62] by adding a known volume of the saturated solution to an excess of standardized $Ba(OH)_2$ solution[63] containing $BaCl_2$. The resulting solution was back-titrated against standardized HCl using phenolphthalein as indicator. Solutions of lower concentrations were prepared by dilution of the stock solution.

Solutions of $NaHCO_3$ were prepared by carefully weighing the anhydrous salt and dissolving it in an 80% ethanol:water solution whose ionic strength was maintained constant at 0.2 M by addition of the appropriate amount of $NaClO_4$.

Since neither CO_2 nor HCO_3^- have easily monitored spectral properties, well established indicator techniques are used[21,28,50,64]. The change in $[H^+]$ accompanying CO_2 hydration or HCO_3^- dehydration is monitored by observing the absorbance change of an indicator anion (A^-) whose response to $\Delta[H^+]$ is rapid compared to the reaction studied (Eq. 14).

$$AH + HCO_3^- \xrightleftharpoons{fast} HCO_3^- + H^+ + A^- \xrightleftharpoons[k_r]{k_f\ (slow)} H_2O + CO_2 + A^- \quad (14)$$

Appropriate buffering conditions were employed (2.5 x10^{-2}M HEPES) such that during the course of the reaction the pH change was less than 0.05 units. Under these conditions the reaction reduces to a typical pseudo first order equilibrium reaction: $HCO_3^- \xrightleftharpoons[k_r]{k_f} CO_2$, and the rate of change in [HCO_3^-] (or rate of change in (A) is given by Eq. 15:

$$\frac{-d[HCO_3^-]}{dt} = \frac{d[CO_2]}{dt} = \frac{d[A^-]}{dt} = k_{obs}[X_e-X] = (k_f + k_r)[X_e-X] \quad (15)$$

where X and X_e stand for the concentration of the product of the reaction at time t and equilibrium respectively[51].

Typically, an experiment was performed by placing a solution consisting of 1x10^{-3} M indicator (bromocresolpurple), 5x10^{-2} M HEPES, enough NaOH to obtain the desired pH, and the appropriate amount of $NaClO_4$ to maintain a constant ionic strength of 0.2 M in one drive syringe. The second drive syringe contained a solution of bicarbonate brought to an ionic strength of 0.2 M with $NaClO_4$. The solvent in both syringes was 80% ethanol:water. After rapid mixing of equal volumes of both solutions at 25°C, reaction rates were monitored by observing the change in [BCP^-] at 580 nm. Control

experiments in which no $NaHCO_3$ was added to the second syringe showed no change in [BCP^-] other than that attributable to dilution after initial rapid mixing thus indicating that the various acid-base equilibria were established rapidly relatively to the reaction under study.

To assess the effect of added catalyst on k_{obs}, different concentrations of the phosphines and equimolar $Zn(ClO_4)_2$ were introduced into the buffer containing syringe. Catalyst solutions were used immediately after preparation since they exhibit diminished activity on standing for prolonged periods, this can be attributable to decomposition of the phosphine as discussed in the Appendix.

In order to obtain a value for the forward rate constant, initial rate experiments were made monitoring the reaction in both directions. For the dehydration reaction the rate of disappearance of bicarbonate ion or appearance of indicator anion can be expressed as in Eq.18a where $d(Abs_{580})/dt$ is the rate of change in measured absorbance at 580 nm and $d[H^+]/d(Abs_{580})$ is an experimental buffer factor relating the changes in absorbance to changes in $[H^+]$.

$$\frac{-d[HCO_3^-]}{dt} = \frac{-d[H^+]}{dt} = \frac{d[A]}{dt} = \frac{d(Abs_{580})}{dt} \cdot \frac{d[H^+]}{d(Abs_{580})} \qquad (18a)$$

Since the solutions employed in this study are highly organic in nature, and the concentration of indicator is large enough to have substantial buffering capabilities, the buffer factors were determined under experimental conditions by rapidly mixing the buffered indicator solution and a second solution containing known $[HClO_4]$ in 80% ethanol:water, 0.2 M in ionic strength and then determining the final absorbance. Plots of $[H^+]$ vs. Abs_{580} are linear over the range $1x10^{-4}$ to $1x10^{-3}$ M in $[H^+]$, the slope being the experimental buffer factor. Initial rates were then determined under identical conditions by measuring the rate of change in absorbance at 580 nm over the first 5% of the reaction. Eq. 18b gives the evaluation of k_f.

$$k_f = \frac{d(Abs_{580})/dt \quad \cdot \quad d[H^+]/d(Abs_{580})}{[NaHCO_3]_i} \tag{18b}$$

To confirm the possible formation of a transient intermediate in the presence of bicarbonate that rendered the evaluation of k_f as meaningless, transient visible absorption spectra vs. time were obtained by stopped flow measurements which give absorbance vs. time plots as a function of λ^{65}. Typically a buffered solution containing BCP and Zn(II) would rapidly be combined with one containing HCO_3^- and the absorbance changes for the first

few seconds of the reaction would be monitored at different wavelengths.

To assess the effect of inhibitory anions, their Na^+ salts were introduced into the syringe containing buffer, indicator and ligand and values of k_{obs} were determined as above.

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APPENDIX

The imidazole phosphine compounds used in the present study are known to be acid sensitive. Unpublished observations by R.S. Brown indicate that when tris(N-(ethoxymethyl)-2-imidazolyl)phosphine is refluxed with HCl, P-C bond cleavage occurs giving imidazole as the product. The mechanism of this process is believed to involve initial protonation of the imidazole which eventually leads to cleavage of the P-C bond. This observation led Brown and co-workers to develop an easily removed protecting group for imidazole[46].

Other observations of the phosphine compounds indicated that the catalytic activity observed for $HCO_3^- \rightleftharpoons CO_2$ equilibration changed as a function of time. It was noticed that when solutions stood overnight, the observed catalysis decreased and reproducible results could only be obtained with freshly prepared solutions.

An NMR experiment was thus performed in order to follow the possible decomposition of the ligands that could account for the observed diminished activity. Ligand 5 was chosen to perform the experiment since the X-ray structure of a crystal obtained by slow diffusion of an ethanol solution of the compound to a water solution of $ZnCl_2$ showed the formation of a ligand:zinc complex in which the phosphorous has oxidized with expulsion of one

of the imidazole substituents. (Figure 15).

The observed compound is a derivative of phosphinic acid ($H_2P(O)OH$) and could presumably be formed in two steps (Eq. 23)[55].

$$RR'R''P \xrightarrow{[O]} RR'R''PO \xrightarrow[\text{or } H_2O]{OH^-} R'R''PO_2H + R^- \qquad (23)$$

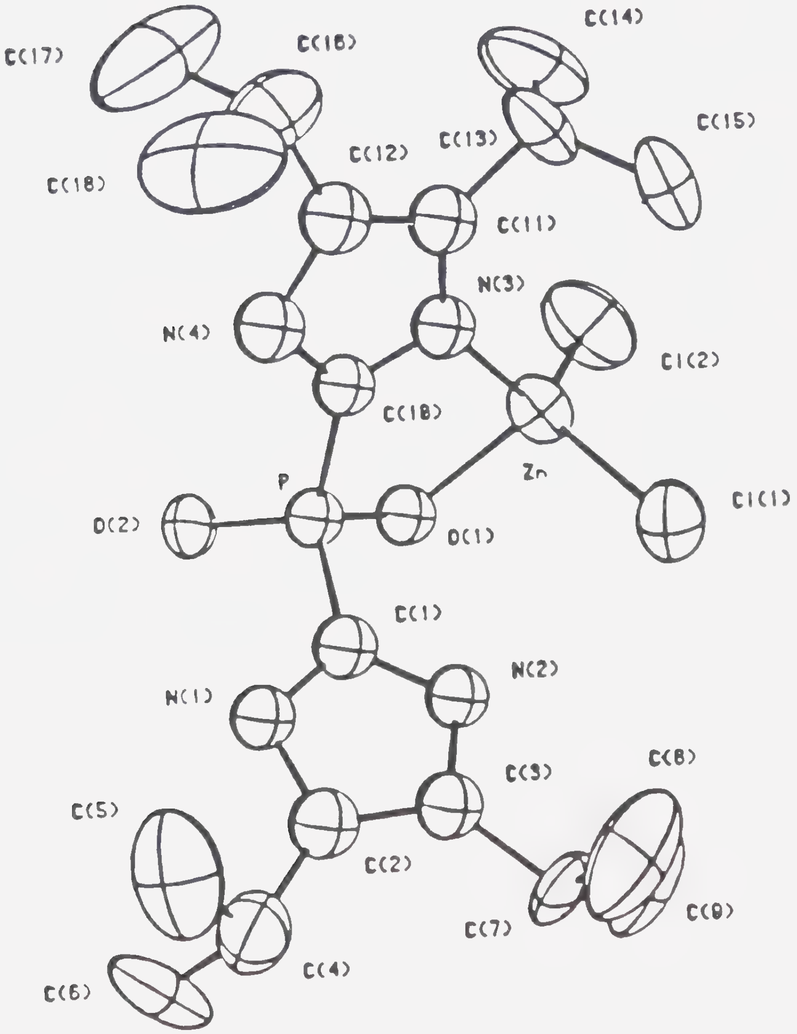
The first step could be the facile oxidation of the phosphine which can then be nucleophilically attacked by either hydroxide ion or water with concomitant expulsion of the best leaving group.

As stated before the decomposition of the imidazole phosphines is a process catalyzed by acid and when zinc ion complexes with the ligand this could have a polarizing effect that could lead to a decomposition similar to the one observed with H^+.

In order to assess this, a solution of 2.5×10^{-2} M HEPES in 80% ethanol:water was used as solvent for 5. Typically 4.4 mg of the ligand were dissolved in 0.5 ml solution and an NMR spectrum was taken at different time intervals. To assess the effect of the zinc, solutions of $ZnCl_2$ and $Zn(ClO_4)_2$ were prepared in D_2O and enough was added to the ligand solutions in order to have a 1:1 ligand:metal ratio. The 9-6 ppm region was monitored. for any signal corresponding to an imidazole 2-H that would indicate decomposition.

FIGURE 15

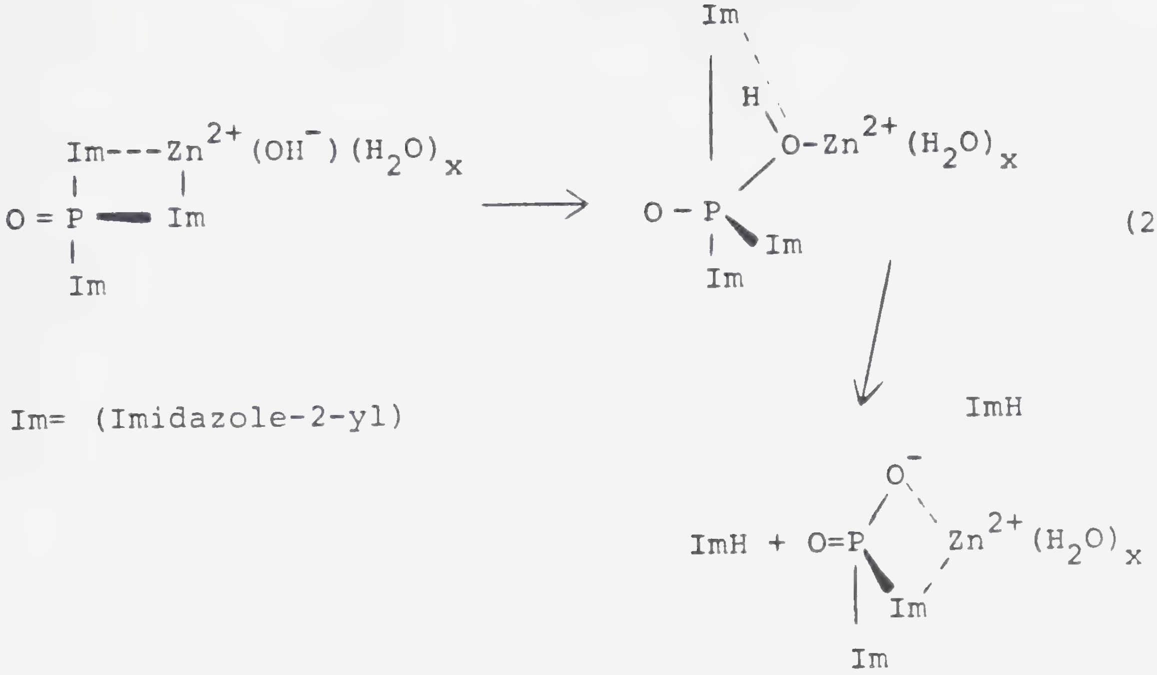
X-ray structure of Bis-(4,5-di-iso-propyl-2-imidazolyl) phosphinic acid:$ZnCl_2$


C(17)
C(16)
C(14)
C(15)
C(18)
C(12)
C(13)
C(11)
N(3)
N(4)
Cl(2)
C(10)
P
Zn
O(2)
O(1)
Cl(1)
C(1)
N(2)
N(1)
C(8)
C(3)
C(5)
C(2)
C(9)
C(7)
C(4)
C(6)

The ligand containing solution showed only one sharp signal in the 9-6 ppm region corresponding to the 4 and 5 hydrogens of imidazole, this remained invariant with time. The ligand:$ZnCl_2$ solution showed one broad signal at 7.4-7.5 ppm corresponding to the 4,5 protons of imidazole; however, after 48 hours a new peak started to develop downfield from the original signal at 7.7 ppm which was interpreted as a 2-H from an imidazole that had been displaced from the phosphine.

Conversion of phosphines with good leaving groups and phosphine oxides into their corresponding phosphinic acids has ample literature precedent[67]. In general, the departing alkyl group is the one having the lower pKa and therefore more capable of supporting negative charge. This would explain why it is the simple imidazole rather than a di*iso*propyl imidazole that is cleaved from 5.

The decomposition of 5 in the absence of Zn^{2+} proceeds very slowly (except under highly acidic conditions) so that the metal ion is in some way catalyzing the process. This could involve a Lewis acid association of Zn^{2+} and imidazole to assist the latter's leaving and/or nucleophilic attack of a chelated Zn^{2+} (OH^-) on the phosphorous as in equation 20.


Im---Zn^{2+}(OH$^-$)(H$_2$O)$_x$
O = P
Im
Im
H
O-Zn^{2+}(H$_2$O)$_x$
O – P
Im
Im
Im
ImH
O$^-$
ImH + O=P
Zn^{2+}(H$_2$O)$_x$
Im
Im

(20)

Im= (Imidazole-2-yl)

B30379